EVALUATION OF A NOVEL APPROACH TO PROMOTING POST-ISCHEMIC RECOVERY OF UPPER EXTREMITY FUNCTION IN THE RAT

BY

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A Thesis
Submitted to the Graduate Faculty
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Biomedical Sciences
Faculty of Veterinary Medicine
University of Prince Edward Island

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Department/Discipline: Biomedical Sciences

Degree: Doctor of Philosophy (PhD) **Year:** 2013

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ABSTRACT

Following stroke, impairments in arm function are common motor deficits in survivors, affecting thousands of people each year. A useful technique used in clinical rehabilitation of patients with arm impairments is to force use of the impaired arm through constraint of the unaffected (or less affected) one. The success of this 'constraint induced movement therapy' (CIMT) is believed to be due to neuroplastic changes that take place on a cellular level in surviving brain tissue, however, little is understood about the mechanisms involved. Appropriate animal models are necessary to study how rehabilitation affects neuroplasticity. Previous literature has described several models of forced use following stroke in rats which have resulted in varying success. Animal stress and lack of behavioural pressure may have contributed to the inconsistency of prior forced use models.

The purpose of the research presented in this thesis was to optimize a surgical model of post-ischemic upper extremity impairment, determine whether it would be possible to force use of the impaired forelimb using a novel appetitively motivated protocol, and then to investigate the effects of this novel model on markers of neuroplasticity. First, the endothelin-1 (ET-1) model of focal unilateral ischemia was optimized by attempting a previously unpublished protocol of injections along the motor cortex and to the striatum. Male Sprague Dawley rats were subjected to ET-1 or sham surgery. Ensuing forelimb functional deficits were measured using a battery of behavioural tests, which were compared to intact sham surgical control performance. The stroke model resulted in reliable and reproducible lesions to forelimb motor regions of the brain, and deficits that lasted up to the end of the 21 day study period in some tests.

Next, this ET-1 stroke surgery was used to evaluate a novel form of forced use rehabilitation in which rats engaged the impaired limb to move voluntarily in commercial pet activity balls. Animals were subjected to ET-1 or sham surgery, and then received either rehabilitation or a control treatment. Behavioural tests revealed that animals receiving rehabilitation recovered to sham levels of performance sooner than animals receiving the control treatment. Stroke, but not rehabilitation, affected the proportion of cells expressing brain derived neurotrophic factor (BDNF) and the presence of doublecortin-positive neuroblasts, but had no effect on the expression of the growth inhibiting protein $NOGO_A$.

Finally, the novel forced use model was developed further to more closely resemble clinical CIMT with the addition of a task-specific reaching component. Animals were subjected to either ET-1 followed by rehabilitation, ET-1 followed by a control treatment, or sham surgery. Again, behavioural tests revealed that animals that had undergone ET-1 surgery had significant deficits that recovered sooner in the group that received rehabilitation. Rehabilitation did not affect the proportion of BDNF-expressing cells, but did appear to cause a shift in the cellular origin of the BDNF that was present. Further, rehabilitation resulted in more doublecortin-positive cells in the damaged hemisphere. This novel approach to rehabilitation represents a useful model of forced use therapy which results in accelerated functional recovery following ischemic injury. The mechanisms underlying this effect may be related to changes in BDNF expression and increased generation or survival of new born cells.

ACKNOWLEDGEMENTS

Throughout my graduate studies, I have been lucky to receive support from several mentors and friends. Since I began my program, my co-supervisors Dr Tasker and Dr Doucette have been incredibly supportive of all of my endeavours and adventures, both academic and extracurricular. I have had the freedom to travel, to present my research in an international forum, and to create and develop Let's Talk Science UPEI, all of which has had an incredible impact on my graduate school experience, as well as my career development.

When I decided to embark on this path in 2008, I thank Dr Jennifer Slemmer and Dr Marva Sweeney-Nixon for their support in this decision, and for the ongoing support I receive from them today. Working in Dr Tasker's laboratory, I have had the opportunity to study under the supervision of an exceptional mentor, while surrounded by supportive people. From the beginning of my program, Dr Tasker has provided me with crucial guidance and ample opportunities to learn. Every member who has been part of our lab group over the years has contributed something positive toward my experience. Specific assistance in this thesis work was provided by Andy Hume, who began the development of the animal model, Carolyn MacKenzie, Melissa Perry, Nathan Marriott, and Emily McGuire, who provided valuable technical assistance, Debra MacDonald, who provided technical training and support, and Drs Daphne Gill and Anabel Perez-Gomez, who provided extensive insight and advice. Without their assistance, I truly would not have accomplished all that I did.

I thank my supervisory committee members: Dr Glenda Wright, Dr Denis Kay, and Dr Harold Robertson, who continue to tirelessly assist me with my ongoing career endeavours. Thank you to Dr Jackalina Van Kampen, for her generous assistance and the use of her equipment. Members of the Atlantic Stroke Group, especially Dr Gail Eskes and Dr Shaun Boe, helped me to appreciate the clinical aspect of my work and inspired my interest in translational research. The staff of the UPEI animal facility, including Chris McQuaid, Zoe Little Grutzner, and Dr Jonathan Spears, has provided valuable assistance with the animal research performed in this thesis.

My family and friends have been tremendously supportive throughout my graduate program. To my husband, Leighton Thomas, thank you for your unwavering support and confidence in me for the last five years, and now, as we look toward the future. To my family: Sara, Paul, Craig, Lois, Jeremy, and Helen, thank you for your support during busy times in all our lives. Thank you Kate and Linda, for keeping me inspired and motivated. To all of you with whom I have had the privilege of sharing an office over the last 5 years: thank you for everything. Especially to Kate, Janet, Amber, Michelle, Bob, Emily, and Priyanka: we had some great times and made memories that I will treasure.

I gratefully thank the several funding sources that supported my graduate program. Innovation PEI, Neurodyn, Canadian Stroke Network, Atlantic Center for Comparative Biomedical Research, UPEI Hooper Klarenbach Scholarship, and the Department of Biomedical Sciences provided financial support that made it all possible.

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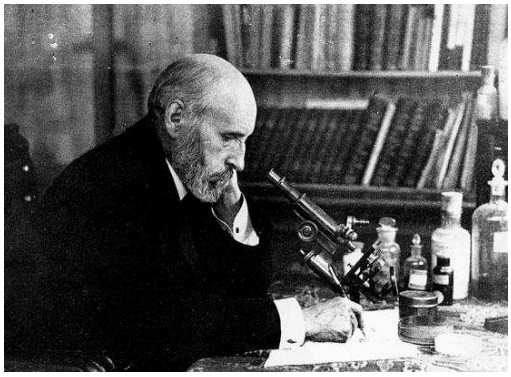
ABBREVIATIONS

ABC	avidin biotin complex
ADL	activities of daily living
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
BSA	bovine albumin serum
CIMT	constraint induced movement therapy
CNS	central nervous system
CPG	central pattern generator
CREB	cAMP response element binding protein
DAB	3, 3'-diaminobenzidine tetrahydrochloride
Dcx	doublecortin
EE	enriched environment
EGF	epidermal growth factor
EPO	erythropoietin
ET-1	endothelin-1
fMRI	functional magnetic resonance imaging
GDNF	glial cell-line derived neurotrophic factor
IGF	insulin growth factor
iNOS	inducible nitric oxide synthase
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
PBS	phosphate buffered saline
PI3K	phosphotidylinositide 3-kinase
PLCγ	phospholipase C gamma

PSD	post surgical day
RM ANOVA	repeated measures analysis of variance
ROCK	Rho kinase
ROI	region of interest
RTP	repetitive task practice
SC	stem cells
SCF	stem cell factor
SGZ	subgranular zone
SVZ	subventricular zone
TBI	traumatic brain injury
TFP	tactile-stimulated forelimb placing
TIA	transient ischemic attack
TNF α	tumour necrosis factor alpha
Trk B	tropomyosin-related kinase B
tPA	tissue plasminogen activator
VEGF	vascular endothelial growth factor
VFP	vibrissae-stimulated forelimb placing

CHAPTER 1

GENERAL INTRODUCTION



"...Once development was ended, the founts of growth and regeneration ... dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh *decree."*

-Santiago Ramón y Cajal (1852-1934)
picture from http://spin.udg.edu/rn12/santiago-ramon-y-cajal/

OVERVIEW

For much of the 20th century, the words of the revered 'father of neuroscience' Santiago Ramón y Cajal (see page 1) were the dogma taught to generations of neuroscientists (Cajal, 1991). Fortunately, critical work in the last few decades has revealed the capacity of the brain to undergo regenerative processes, prompting a surge in research into neurorehabilitation of the nervous system following injury and disease.

Stroke is a leading cause of death and disability (Hootman et al., 2009; Statistics Canada, 2000). While preventive therapies and lifestyle modifications can reduce the incidence of stroke (Bousser, 2012), countless people live with the devastating disabilities associated with the disease and require some form of rehabilitation to recover. A particularly common and pervasive disability in survivors of stroke is decreased sensorimotor function of the contralateral arm (Kelly-Hayes et al., 1998). An increasingly popular rehabilitative technique that is being developed to increase use and improve function of the impaired arm is constraint induced movement therapy (CIMT) (Nijland et al., 2011). During CIMT, the affected arm is constrained, forcing patients to use their impaired arm. CIMT has been shown to improve functional outcome (Bonaiuti et al., 2007; Nijland et al., 2011; Sawaki et al., 2008; Wittenberg et al., 2003; Wolf et al., 2011), even when administered to patients with chronic deficits (Liepert et al., 2000).

The mechanisms underlying CIMT-associated improvements in recovery are not well understood (Sawaki et al., 2008). This has prompted interest in modelling 'forced use' rehabilitation in animals in order to more closely examine resulting changes in the brain. To date, animal models of forced use therapies have had varying success at

producing functional improvements. The purpose of the work described in this thesis was to develop and evaluate the efficacy of a novel animal model of voluntary forced use on recovery of forelimb function, and to investigate resulting alterations in the expression of markers associated with neuroplasticity in the brain.

1.1 STROKE

Approximately 850,000 North Americans are clinically diagnosed with stroke each year (Heart and Stroke Foundation, 2012; Hootman et al., 2009; Roger et al., 2011; Statistics Canada, 2000) and many more may experience transient ischemic attacks (TIAs), known as 'silent strokes' (Roger et al., 2011). In addition to a high mortality rate, the majority of survivors are left with motor disabilities (Heart and Stroke Foundation, 2012). Patients can remain chronically impaired for months to years following a stroke, which vastly impacts quality of life and is associated with a higher rate of post-stroke depression (Burvill et al., 1997; Ramasubbu et al., 1998). As a result, it has been suggested that stroke has a greater disability impact than any other chronic disease (Adamson et al., 2004).

There are two types of stroke: ischemic, which involves vessel occlusion, and hemorrhagic, associated with vessel rupture. At least 80% of strokes diagnosed in North America are ischemic (Heart and Stroke Foundation, 2012; Roger et al., 2011). Occlusion affecting blood supply to the entire brain results in global ischemia, while focal ischemia occurs when only part of the brain is affected. Ischemic episodes may be permanent or temporary, wherein dissolution of the clot leads to a period of reperfusion to the affected area. During the ischemic episode, lack of oxygen exchange to the

affected region ultimately leads to the development of an infarct (Hertz, 2008), as described below. Because of its high prevalence, temporary focal ischemia is the focus of the work in this thesis.

1.2 THE PATHOLOGY OF ISCHEMIA

Once vessel occlusion occurs, a complex series of multiple cellular events is rapidly set in motion, as described below (summarized in Figure 1.1; numbers on Figure correspond to description below) (Hertz, 2008; Teasell et al., 2005). The brain is comprised of several cell types, including neurons, astrocytes, oligodendrocytes, and endothelial cells. Each of these is vulnerable to the effects of ischemia, albeit to varying degrees (Denes et al., 2010), and together they comprise an intricately intertwined network system. Hence, when a subpopulation of cells is injured, the effects are not constrained to a particular cell type or group; there will be ramifications to the surrounding microenvironment, and to remote connected regions.

Initially, lack of blood (and, therefore, oxygen and glucose exchange) results in energy depletion, which impairs cellular ion pumps (1) leading to cell depolarization (2). If energy depletion is excessive, both apoptotic and necrotic pathways are initiated and cell death occurs (3) (Lee et al., 2000). Both depolarization and cell death lead to the release of glutamate, an excitatory amino acid, into the surrounding extracellular space, while glutamate sequestering systems are disrupted (4). Glutamate can then stimulate surrounding cells, resulting in N-methyl-D-aspartate (NMDA) and non-NMDA receptor-mediated excitotoxicity (5) (Durukan and Tatlisumak, 2007; Lee et al., 2000). Excitotoxicity results in massive calcium (Ca^{2+}) influx, mitochondrial dysfunction,

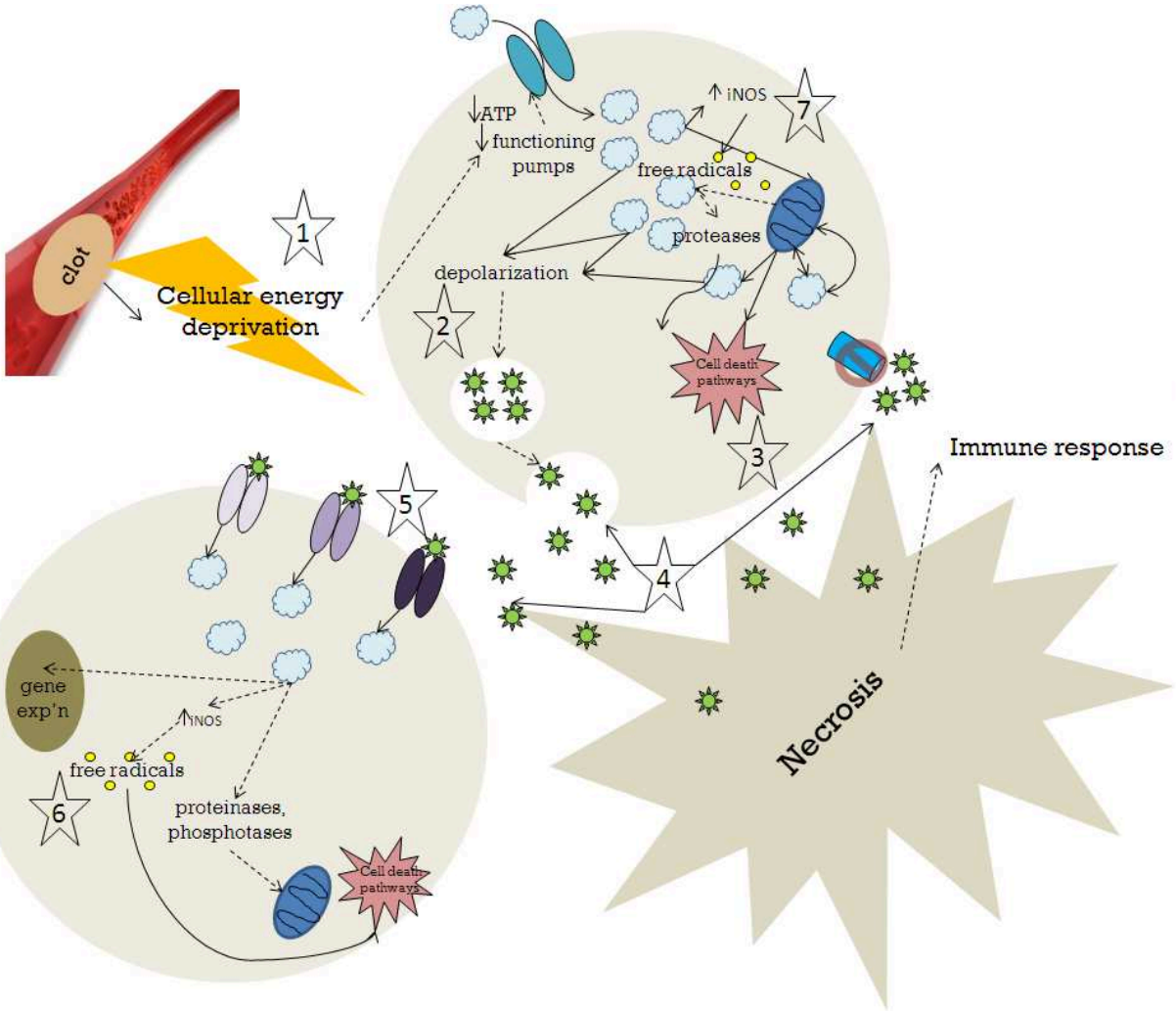


Figure 1.1. General overview of pathological events in the ischemic cascade. In this example, a blood clot results in reduced transport of oxygen and glucose, which results in cellular energy deprivation and a number of downstream effects involving Ca^{2+}, free radical production, and excitotoxicity, and can result in apoptosis, necrosis, and altered gene expression. See text for stepwise description of numbers. Figure created by J. Livingston-Thomas, from information contained in references cited in Section 1.2.

and the formation of reactive oxygen species, which inhibit energy production and attack cellular components (6). Moreover, induction of inducible nitric oxide synthase (iNOS) leads to increased release of nitric oxide (NO) which enhances oxidative damage and can contribute to further cell death (7) (Forman et al., 1998).

Because of the self-perpetuating and outwardly spreading nature of this process, the damage that occurs following stroke evolves over time, initially consisting of a necrotic region resulting from the death of cells in the immediately affected area (the core), and a surrounding peri-infarct region (the penumbra). In the core, the excitotoxic overload and bio-energetic depletion described above results in immediate necrotic and apoptotic cell death, while the penumbral region experiences moderate sub-lethal ischemia, due to less-intensive damage or partial blood supply from collateral vessels (Hertz, 2008). While this moderate reduction in blood flow can become sufficient to cause the loss of electrical excitability, these cells can maintain membrane potential and ion gradients temporarily. The penumbra is considered reversibly compromised, because tissue can be affected by further damage spreading from the core but is also sensitive to appropriate interventions (Hossmann, 1994). Damage in the penumbra can occur for up to three days following the ischemic incident (Hata et al., 2000; Hossmann, 1994). The timing of the spectrum of ischemic cascade events varies depending on infarct size, duration of ischemia, and timing and effectiveness of reperfusion, if applicable. Figure 1.2 generalizes the events into those that occur within minutes, hours, and days.

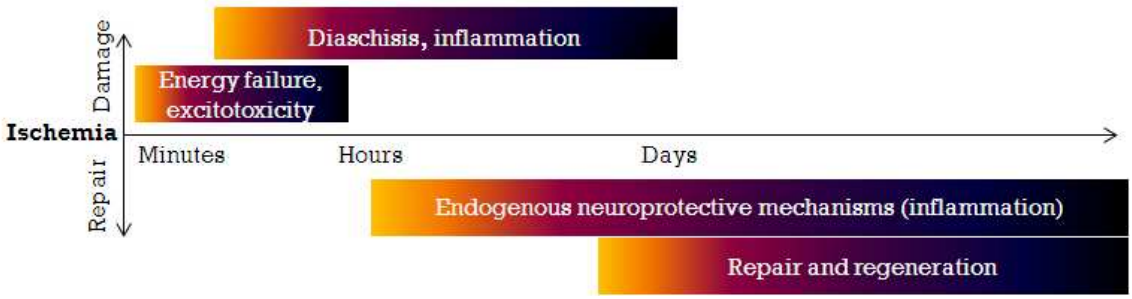


Figure 1.2. Timeline of events following ischemia. Ischemia results in the initiation of both protective and detrimental processes. These are generalized into those that take place within minutes, hours, and days. Immediately, energy failure and subsequent excitotoxicity occur. Secondary damage processes including edema and diaschisis peak after hours. Eventually, a dynamic growth environment results in repair processes, including regeneration of axons and dendrites and formation of synapses. The role of inflammation is complicated, and believed to include both damaging and reparative processes, therefore it appears on both sides of the spectrum. Figure adapted from (Endres et al., 2008; Wieloch and Nikolich, 2006).

1.3 POST-ISCHEMIC MOTOR DEFICITS: MECHANISMS OF RECOVERY

The middle cerebral artery (MCA) is most commonly affected in ischemic stroke (Ng et al., 2007). The MCA supplies blood to areas of the cortex and striatum, including the primary motor and sensory areas of the hand and arm (Traystman, 2003). Disruption of blood flow to these regions largely results in motor and sensorimotor deficits in contralateral forelimb function (Feys et al., 1998; Modo et al., 2000). These impairments are common in survivors of stroke (Kelly-Hayes et al., 1998).

Initial post-stroke impairments are usually followed by some degree of spontaneous (unaided) recovery, after which remaining deficits may last years without further improvement. Generally, the degree of spontaneous recovery is minor, and occurs soon after the initial insult. Spontaneous recovery in humans is partly attributed to the resolution of secondary injury processes that are produced by the initial trauma, and which resolve within the first days and weeks following ischemia (see Figure 1.2). These include edema, diaschisis, and inflammation, described below. The degree of both spontaneous and later long-term recovery is highly variable, and depends on the severity of the infarct and demographic characteristics such as age, gender, and prior health status (Braeuninger and Kleinschnitz, 2009; Huang et al., 2012, 2009; Johansson, 2000).

Two forms of edema normally follow ischemic injury. Cytotoxic edema occurs due to cellular swelling in response to ionic imbalance resulting from ischemia (Durukan and Tatlisumak, 2007; Hossmann, 2008), while vasogenic edema occurs due to the breakdown of the blood brain barrier (Hossmann, 2008; Watanabe et al., 1977a). Edema gradually resolves over the first days following injury, restoring some function to

affected neurons (Teasell et al., 2005). Diaschisis is the state of depressed neurological function resulting from the interruption of input from remote but neuroanatomically connected brain regions. Resolution of diaschisis within the first hours and days following the insult can result in the return of neurological function to affected areas (Teasell et al., 2005). Inflammation, which is initiated soon after onset of ischemia, may play several roles. Microglia and other cell types involved in the immune response are believed to have both harmful (Iadecola and Anrather, 2011) and beneficial effects on the post-injury brain (Faustino et al., 2011; Iadecola and Anrather, 2011; Madinier et al., 2009), but in general the resolution of early inflammatory processes is believed to contribute to early spontaneous recovery (Denes et al., 2010).

Humans experience little short-term recovery before reaching a plateau at which remaining deficits may be chronic (Krakauer, 2005). However, in rodent models of stroke, unassisted recovery occurs more quickly, and can result in more complete functional recovery, depending on the evaluation used. For example, reflexive and gross motor deficits often recover to baseline levels within one month without intervention (Hume, 2009; Leasure and Grider, 2010; Schallert et al., 2000a), whereas dextrous motor function remains impaired for much longer or does not recover within the reported time frame (Biernaskie and Corbett, 2001; Ploughman et al., 2009; Soleman et al., 2010).

1.4 NEUROPLASTICITY

Once considered a largely static organ following development, the adult brain is now known to be structurally and functionally dynamic. The ability to reorganize by

forming and sculpting new neural connections throughout life is a phenomenon called 'neuroplasticity'. In the healthy adult brain, much of this plasticity occurs during learning and memory processes. However, following injury to the nervous system including stroke, post-ischemic neuroplastic processes are initiated. A better understanding of these inherent repair mechanisms could result in substantial advances in post-stroke recovery.

1.4.1 Neuroplasticity in the injured brain

Injury to the central nervous system is a potent trigger of plasticity (Chopp et al., 2008; Ohab et al., 2006; Schallert et al., 2000b). In human patients, examination of motor maps by functional magnetic resonance imaging (fMRI) reveals altered activation patterns following stroke that evolve over time. For example, during a particular motor task that normally requires the brain region that is damaged, patients with poor initial recovery are more likely to recruit brain regions in the contralesional hemisphere. As more complete recovery is observed, there is increased activation of ipsilesional brain areas (Bastings et al., 2002; Cramer, 2004; Teasell et al., 2005; Ward et al., 2003). A similar positive correlation between recovery and ipsilesional activation is observed in rats (Dijkhuizen et al., 2003). Such alterations in functional activity maps are attributed to the recruitment of previously unrelated pathways in the brain, and to neuroplastic processes that take place on a cellular level (Nudo et al., 2001). These processes include axonal regeneration (Carmichael and Chesselet, 2002; Carmichael et al., 2005; Dancause et al., 2005), dendritic growth (Hsu and Jones, 2006; Jones and Schallert,

1992), and neurogenesis (Jin et al., 2001; Leasure and Grider, 2010; Ohab et al., 2006; Wang et al., 2007).

1.4.2 Regeneration

As discussed previously, following ischemic stroke, a necrotic core of tissue develops which is surrounded by the penumbra (Hossmann, 2008). Cells in the penumbra retain the capacity to sustain membrane potentials, while being subjected to spreading glutamate excitotoxicity and other pathological processes (see Section 1.2). Elevation of intracellular Ca^{2+} concentrations in these cells ultimately results in the modulation of gene expression by affecting the phosphorylation of transcription factor cAMP-response-element-binding protein (CREB) (see Figure 1.1) (Di Filippo et al., 2008; Ohab et al., 2006). Various genes can be affected, including pro- and anti-apoptotic genes, as well as those involved in inflammation, neuroprotection, and cell growth (Li and Carmichael, 2006; Raghavendra Rao et al., 2002) Consequently, ischemia can lead not only to cell damage and death, but alternatively, to a dynamic growth-modulating microenvironment.

Post-ischemic neuroplastic changes have been described in both the injured and intact hemispheres. In ipsilesional tissue, there is an immediate degeneration of axons, dendritic spines, and synapses in the first days following injury, followed by axonal sprouting as early as the third day post-injury and synaptogenesis 1-2 weeks later persisting up to 12 weeks (Ito et al., 2006; Nudo et al., 2001). Similarly, in the contralesional hemisphere, dendritic overgrowth begins in the first few days following injury, followed later by dendritic pruning and increased synapse formation (Jones and

Schallert, 1992). By 30 days post-injury, there are more multiple synaptic boutons and perforated postsynaptic densities (Kleim et al., 2003; Nudo et al., 2001). These structural changes at a cellular level underlie the brain's ability to reorganize, and presumably, to recover function.

1.4.3 Growth-associated proteins

Ischemia alters the expression of several genes (Mizutani et al., 2011; Raghavendra Rao et al., 2002), including those associated with cellular growth, division, and apoptosis. Carmichael et al. (2005) performed a detailed analysis that demonstrated the temporally-defined patterns of expression of various genes over the first month post-ischemia (Carmichael et al., 2005). Later termed 'gene expression waves' (Carmichael, 2006), the patterns of several of these growth-associated genes are summarized in Figure 1.3 (Carmichael et al., 2005; Li and Carmichael, 2006). This gene regulation is linked to the differential expression of various proteins involved with promoting and inhibiting growth (Abe, 2000). Of particular relevance to this thesis are brain derived neurotrophic factor (BDNF), and the growth-inhibitory protein $NOGO_A$.

1.4.3.1 Brain derived neurotrophic factor

Growth factors are proteins that have the capacity to stimulate cell growth, differentiation, and proliferation. Neurotrophins are a family of growth factors that influence the survival of neurons. BDNF, one of the most intensively studied of these, has been implicated in post-ischemic neuroplastic events, described in more detail

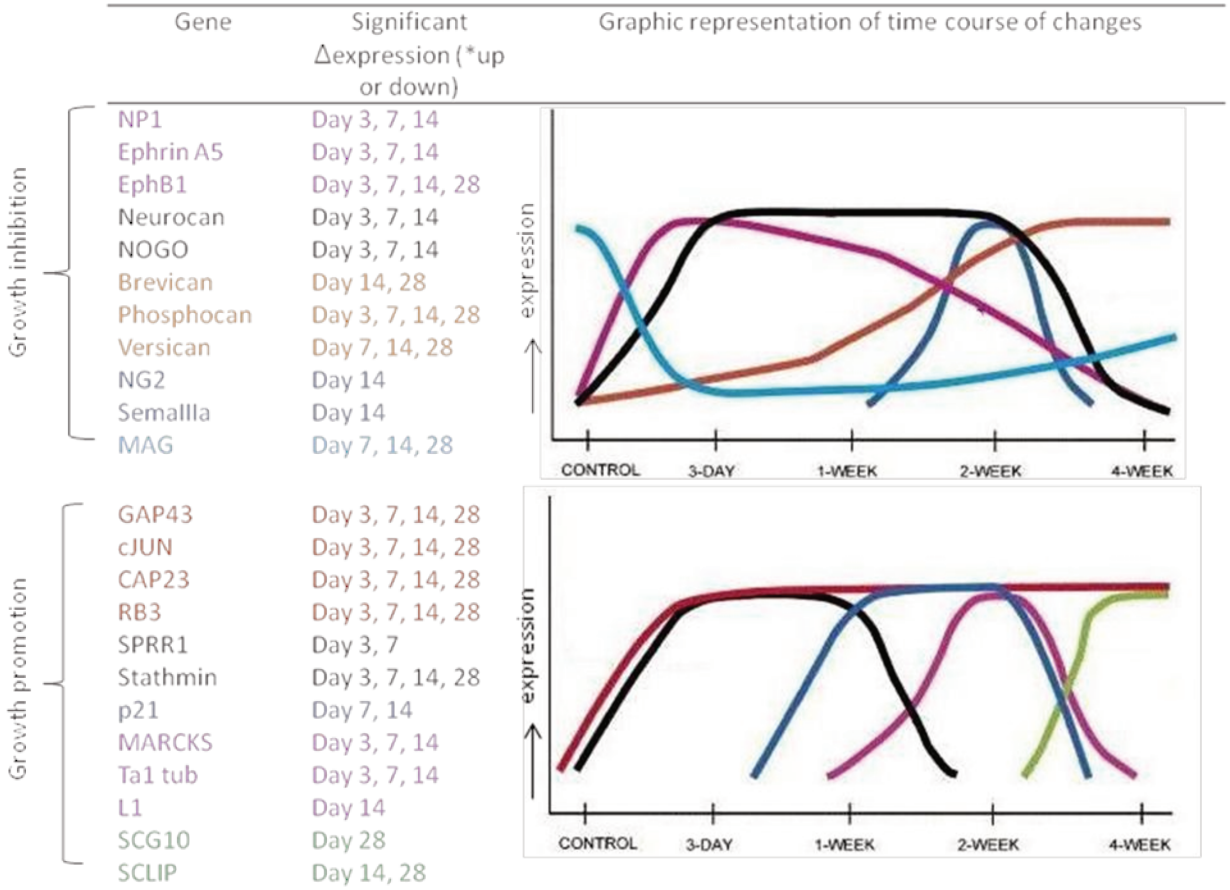


Figure 1.3. Differential gene regulation following stroke. The gene expression of several growth-inhibiting and –promoting proteins have been characterized up to 4 weeks post-stroke, revealing a dynamic temporally dependent expression pattern. Data and graphs adapted from (Li and Carmichael, 2006).

below. Other growth factors, such as the glial cell-line derived neurotrophic factor (GDNF) family, and non brain-specific proteins such as insulin growth factor (IFG)-1 and basic fibroblast growth factor (bFGF)-2, are also implicated in post-ischemic neuroplasticity (Greenberg and Jin, 2006; Horinouchi et al., 2007), although not the focus of the present work.

BDNF belongs to the neurotrophin family, consisting of structurally-related nerve growth factor (NGF), neurotrophin-3, neurotrophin-4/5, all of which modulate cell survival (Lu, 2003). BDNF is a well-characterized neurotrophic factor that has been implicated in numerous post-injury recovery processes including neurogenesis, dendritic growth, and synaptic modulation (Greenberg et al., 2009; Nagappan and Lu, 2005). BDNF expression is activity-dependent (Nagappan and Lu, 2005); it is upregulated during long term potentiation (Greenberg et al., 2009; Lu, 2003; Vaynman and Gomez-Pinilla, 2005) and exercise (Griesbach et al., 2009; Kim et al., 2005; Ploughman et al., 2009). Thus, the increased neuronal activation that takes place in the penumbral zone may modulate BDNF expression (Di Filippo et al., 2008).

Produced as a precursor peptide, pro-BDNF is cleaved primarily by extracellular proteases including metalloproteinases and plasmin. The product is mature BDNF protein, which binds to the tropomyosin-related kinase B (TrkB) receptor (Lu, 2003). TrkB receptor autophosphorylation then activates downstream signal transduction pathways including MAPK, PLCγ, and PI3K. This signalling leads to the activation of transcription factors that can alter expression of a number of growth-associated genes such as those regulating growth associated protein GAP43 (associated with neuronal sprouting), synaptophysin (associated with syntaptogenesis), and further BDNF

expression (Figure 1.4) (Jovanovic et al., 2000; Lu, 2003; Madinier et al., 2009; Vaynman et al., 2004).

There is compelling evidence to suggest that BDNF plays a critical role in post-ischemic reorganization and recovery. Post-stroke interventions that improve functional recovery can be associated with increased BDNF (Ke et al., 2011; Kim et al., 2005; MacLellan et al., 2011), and exogenously administered BDNF facilitates motor recovery (Müller et al., 2008; Schabitz et al., 2004). Conversely, interfering with BDNF by using antisense oligonucleotides (Ploughman et al., 2009) or by blocking TrkB receptors (Griesbach et al., 2009) reduces neuroplasticity and attenuates recovery. However, a study using BDNF +/- mice reported that after permanent ischemia, mice with deficient BDNF production recovered sooner than wild types (Nygren et al., 2006), suggesting a potential detrimental role of BDNF to post-stroke plasticity.

Despite this controversial role in recovery, endogenous post-stroke BDNF expression has been poorly investigated (Béjot et al., 2011; Madinier et al., 2013). Recently, Béjot et al. (2011) found that BDNF expression in both the ipsi- and contralateral hemisphere increased following embolic stroke. Furthermore, several cell types in the brain (neurons, astrocytes, microglia, endothelial, and ependymal cells) were responsible for BDNF expression, emphasizing the potential importance of non-neuronal cells (as well as neurons) in neuroplastic processes.

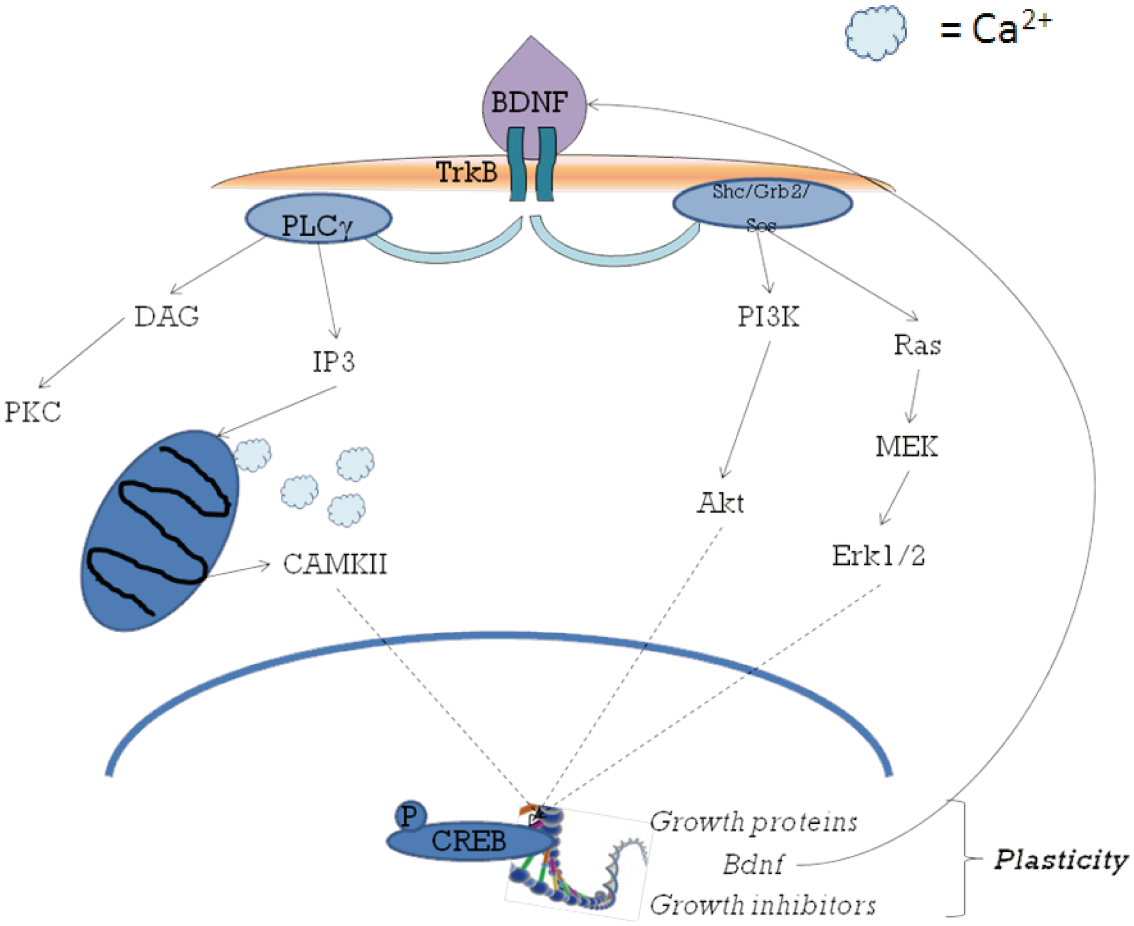


Figure 1.4. Overview of BDNF signalling. BDNF plays an important role in post-ischemic neuroplasticity though interaction with the TrkB receptor and activation of downstream signalling through the Akt, Erk, and CAMKII pathways, ultimately leading to altered gene expression (Vaynman et al., 2004). Dotted lines indicate entry into the nucleus via pores.

1.4.3.2 $NOGO_A$

$NOGO_A$ belongs to a small family of proteins which inhibit neuronal sprouting (Schwab, 2004). $NOGO_A$ binds to the NOGO receptor NgR1, which activates the RhoA pathway and Rho kinase. This leads to microtubule depolymerization and actin contraction, leading to growth cone collapse and failed neural regeneration (Figure 1.5) (Carmichael, 2010).

Following central nervous system (CNS) injury, neuronal $NOGO_A$ expression is increased, which may represent an endogenous attempt to inhibit spontaneous axonal remodelling that could worsen functional recovery (Cheatwood et al., 2008). Cheatwood et al. (2008) performed detailed analyses of $NOGO_A$ expression following middle cerebral artery occlusion (a stroke model described below, in Section 1.6), and reported dynamic changes in $NOGO_A$ expression over time and in various brain regions, including the cortex (Cheatwood et al., 2008). In the ipsilesional hemisphere, the protein was highly expressed in the forelimb sensorimotor cortex immediately following ischemia, and up to 28 days post-injury. In the contralateral hemisphere, there was an initial decrease in $NOGO_A$ expression following ischemia, which returned to baseline levels a week later (Cheatwood et al., 2008).

Using a similar stroke model, Papadopoulos et al. (2002) found that post-ischemic neuroplasticity and functional recovery could be enhanced by neutralizing the effects of $NOGO_A$ using an antibody to the protein. Similarly, following ischemia, Fang et al. (2010) used a $NOGO_A$ antagonist combined with rehabilitation to successfully promote functional recovery. These results suggest that $NOGO_A$ plays an important role in post-ischemic neuroplastic remodelling.

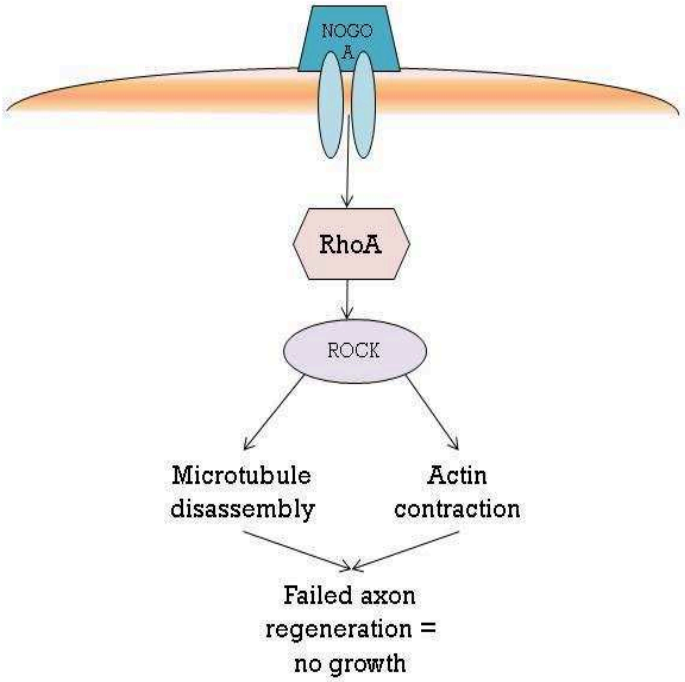


Figure 1.5. Overview of $NOGO_A$ signalling. $NOGO_A$ plays an important role in post-ischemic neuroplasticity though interaction with the Nogo receptor NgR1, which activates RhoA signalling and Rho kinase (ROCK), ultimately blocking axon growth through growth cone collapse (Carmichael, 2010).

1.4.3.3 Neurogenesis

While it has long been recognized that many adult non-nervous system tissues have the capacity to be renewed through the production of stem cells (SC), it was widely accepted that the brain did not possess this ability. In recent decades, the discovery that the adult brain does produce neural SCs represented a major breakthrough in the study of neurodegenerative disease, because of the potential for therapeutic manipulation of this process to promote brain repair (Altman, 1962; Eriksson et al., 1998; Reynolds and Weiss, 1992). In the healthy brain, SCs are constantly being formed in two main regions: the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) adjacent to the lateral ventricles (Lois and Alvarez-Buylla, 1994; Zhang et al., 2001). In the healthy adult brain, the SCs originating in the SGZ become integrated into the granule cell layer, while the SCs originating in the SVZ follow a rostral migratory stream to the olfactory bulb (Lois and Alvarez-Buylla, 1994). Neural SCs are multipotent and give rise to various neural cell types (neurons, astrocytes, and oligodendrocytes) (Momma et al., 2000).

Several studies have shown that ischemia results in reactive neurogenesis in the SVZ, and migration of new cells toward the site of lesion (Gu et al., 2000; Jiang et al., 2001; Jin et al., 2001; Leasure and Grider, 2010; Parent et al., 2002; Wang et al., 2007; Xiong et al., 2010; Zhang et al., 2001). While the role of these endogenous newborn neural cells remains unclear, they likely contribute to functional recovery, because attenuation of neurogenesis by genetic ablation (Jin et al., 2010; Sun et al., 2012) or irradiation (Raber et al., 2004) worsens functional outcome following ischemia.

Evaluations of the efficacy of SC transplantation following stroke are ongoing. Several paradigms have been reported using SCs from various sources, including human umbilical cord blood (Chen et al., 2001), bone marrow (Chen et al., 2003; Li et al., 2001), embryonic tissue (Bühnemann et al., 2006a; Hicks et al., 2009), and hNT cells derived from human teratocarcinoma cell line (Bliss et al., 2006), which have been administered via the blood stream (Chen et al., 2001, 2003; Li et al., 2001) or grafted intracerebrally (Bliss et al., 2006; Bühnemann et al., 2006a; Hicks et al., 2009) [see Smith and Gavins (2012) for review]. Unfortunately, there is evidence that grafted SCs may not exhibit long-term survival, develop normally, express neurotransmitters, or integrate into existing neural networks (Arvidsson et al., 2002; Bühnemann et al., 2006b; Hicks et al., 2009), possibly due to suboptimal experimental conditions. Furthermore, there are a number of additional challenges associated with stem cell transplantation including graft rejection, the use of relatively invasive procedures, and ethical concerns (e.g. if SCs are obtained from human fetal tissue) (Liu et al., 2009). Interestingly, various forms of experimental post-stroke rehabilitation strategies have been shown to enhance endogenous neurogenesis (Hicks et al., 2007; Mizutani et al., 2011; Nilsson et al., 1999; Wurm et al., 2007). However, the long-term fate of newly born SCs, and their contribution to functional recovery, remains unclear (Arvidsson et al., 2002).

1.5 TREATING STROKE

Despite advances in the diagnosis and management of acute ischemic stroke in recent decades, it remains a leading cause of mortality and disability (Hootman et al.,

2009; Statistics Canada, 2000). Because cells in the ischemic core die within minutes (see Figure 1.2), the primary goal of acute stroke therapy is to salvage tissue in the penumbra. The current standard of care involves the use of a thrombolytic compound, recombinant tissue plasminogen activator (tPA), to restore blood flow resulting from a clot. It is presently the only approved drug for administration following stroke, but can only be administered within 4.5 hours of stroke onset (Stemer and Lyden, 2010), and requires prior confirmation that the stroke is ischemic rather than hemorrhagic (Madden, 2002; Stemer and Lyden, 2010). In most cases, this time frame is insufficient for patients to seek medical help, undergo clinical assessment, and be administered the drug. As a result, it has been estimated that fewer than 5% of stroke victims receive tPA (Reeves et al., 2005). These limitations have fuelled the search for other therapeutic options including alternative thrombolytic agents (Balami et al., 2013), adjuvant compounds that could extend the time window for treatment [e.g. atorvastatin; (Zhang et al., 2010)], and neuroprotectants [e.g. PSD95 inhibitors; (Sun et al., 2008)].

More than 1,000 candidate strategies for treating stroke have failed in clinical trials (O'Collins et al., 2006), likely due to the inability of current preclinical models to adequately mimic clinical stroke (Radermacher et al., 2012). Many different therapeutic approaches have been explored, including the use of free radical scavengers, excitatory amino acid antagonists, hypothermia, barbiturates, calcium channel blockers, and growth factors (STAIR, 1999). However, despite the fact that many of these approaches have shown promise in preclinical experimentation, none have been successful in clinical trials (Fisher et al., 2009; O'Collins et al., 2006; STAIR, 1999).

During ischemia, cells are subject to excitotoxic events largely mediated by the binding of excess glutamate to NMDA receptors (see Figure 1.1). Thus, one obvious

neuroprotective strategy is to block NMDA receptors. Unfortunately, conventional extracellular antagonism of NMDA receptor activation results in severe dose-limiting side effects because of the essential role of glutamatergic signalling in the brain. A promising alternative approach currently being explored is to specifically target intracellular NMDA receptor-related cytotoxic events following ischemia without affecting normal function, thereby preventing damage while avoiding detrimental side effects (Aarts et al., 2002; Cook et al., 2012; Sun et al., 2008).

Other alternative approaches to treating stroke include targeting downstream processes such as inflammation (Endres et al., 2008). The role of inflammation following stroke is complicated, and it is believed to have both beneficial and detrimental effects on brain recovery (Denes et al., 2010; Faustino et al., 2011; Iadecola and Anrather, 2011; Madinier et al., 2009). Cytokines such as interleukins (e.g. IL-1β, IL-6) and tumour necrosis factor alpha (TNF)-α are released during inflammation, and have been shown to contribute to brain damage following ischemia (Relton and Rothwell, 1992). Thus, an emerging therapeutic approach to limit damage is to interfere with the inflammatory response, either alone (Pradillo et al., 2012) or as an adjuvant to other pharmacotherapy (Sumbria et al., 2013). While this may represent an effective early strategy, inflammation may play a vital role in assisting neuroplastic processes in the weeks following ischemia (Denes et al., 2010; Madinier et al., 2009; Narantuya et al., 2010) .

While these approaches currently show promise in preclinical studies, the clinical efficacy of such treatments has not been established. Consequently, tPA administration remains the only presently available treatment for those experiencing a stroke. Because of logistical issues described earlier, many patients do not receive tPA and are left with

brain damage and resultant disability. These patients must rely on treatments aimed at improving recovery of function, the further development of which requires clinically predictive animal models.

1.6 ANIMAL MODELS OF STROKE

As described earlier, cellular death and damage in ischemic stroke is a complex process involving a variety of cell types and cellular mediators (see Section 1.2). As such, in-depth investigation into the processes underlying ischemic damage and subsequent functional recovery requires the use of whole animals. Addressing all aspects of the disease using any given animal model may be impossible, but several models have contributed to our understanding of the mechanisms involved. To date, a variety of animal models including pigs (Imai et al., 2006) and non-human primates (Watanabe et al., 1977b) have been explored, but rat models of stroke are the most common choice for researchers. This is largely due to the similar pattern of neurovascular branching to humans (Macrae, 1992; Yamori et al., 1976), relatively low cost (Durukan and Tatlisumak, 2007), and the high number of validated behavioural tests of functional outcome in the rat (Durukan and Tatlisumak, 2007; Macrae, 1992; Tamura et al., 1981). In the case of forelimb rehabilitation studies, rats are especially useful because of the similarity of their limb movement patterns to that of humans (Whishaw et al., 2002).

The following section summarizes the most common rat models of stroke used to study post-stroke rehabilitation, including the model chosen for the present research (summarized in Table 1.1). There are several other models of focal ischemia that are

Stroke Model	Advantages	Disadvantages	References
Devascularization	-Models permanent ischemia; -Relatively good control over lesion size location; -Low mortality rates	-Requires removal of skull tissue; -Mechanical damage can occur to surrounding tissue and vessels; -Can produce surface damage only;	(Gonzalez and Kolb, 2003; Kleim et al., 2007; Metz and Whishaw, 2002; Whishaw, 2000)
Photothrombosis	-Models permanent ischemia; -Low mortality rate; -Precise control over lesion size and location; -Full craniectomy can be avoided	-Can only produce cortical damage; -Penumbra area different from that observed following stroke	(Carmichael, 2005; Carmichael et al., 2004; Markgraf et al., 1993; Müller et al., 2008; Shanina et al., 2006; Watson et al., 1985)
MCAo	-Models transient or permanent ischemia; -Results in cortical and striatal damage -Widely used and well-characterized	-Large and variable infarcts; -Collateral damage to non-targeted vasculature; -Feeding problems may occur; -Some mortality	(Boyko et al., 2010; Gerriets et al., 2004; Lipsanen and Jolkkonen, 2011; Longa et al., 1989)
Endothelin-1	-Models transient ischemia; -Can produce cortical and striatal damage; -Ability to control precise variables resulting in localized lesions; -Can be used to model lacunar infarcts -Low mortality rate	-Requires removal of some skull tissue; -Mechanism of vessel occlusion not well elucidated -Unknown nonspecific effects due to presence of ETR on non-vascular cells	(Adkins et al., 2008; Fuxe et al., 1997; Sharkey et al., 1993; Windle et al., 2006)

Table 1.1. Common animal stroke models. A summary of advantages and disadvantages of commonly used animal models of focal ischemic stroke used in animal rehabilitation research.

widely used for other aspects of stroke research, such as embolization and spontaneous infarction using hypertensive rats (Yamori et al., 1976). There are also a number of models of multi-focal and global ischemia, as well as hemorrhagic models. A detailed discussion of these is beyond the scope of this thesis; for comprehensive reviews see (Hossmann, 2008; Kleim et al., 2007).

1.6.1 Devascularization

Complete ischemia by eradication of blood supply to brain tissue can be achieved by devascularisation. In this approach, vasculature on the surface of the brain is physically removed, for example by rubbing with a cotton swab (Gonzalez and Kolb, 2003; Metz and Whishaw, 2002; Whishaw, 2000), or by electrocoagulation (Humm et al., 1998, 1999; Kozlowski et al., 1996). This method generally produces focal cortical damage that extends from the pia to the white matter (Kleim et al., 2007). However, it does not produce striatal damage that is often associated with clinical stroke (Cramer, 2003). Furthermore, devascularisation cannot model temporary ischemia, a common clinical presentation of stroke. Mechanical damage to surrounding tissue can also occur, resulting in hemorrhage and non-targeted damage (Kleim et al., 2007).

1.6.2 Photothrombosis

Photothrombotic stroke models induce cortical damage by the systemic injection of a photoactive dye (e.g. Rose Bengal) followed by irradiation of blood vessels by a light beam, usually through the skull (Watson et al., 1985). This damages the

vasculature and causes ischemia. This method can be used to produce widespread damage by targeting a major artery such as the MCA (Markgraf et al., 1993; Yao and Nabika, 2011; Yao et al., 2003), or to create a more localized lesion by directly targeting the vasculature in a particular brain area (Müller et al., 2008; Shanina et al., 2006; Watson et al., 1985). Focal photothrombosis allows the experimenter to easily target precise cortical regions, results in relatively reproducible lesions, and is associated with a low mortality rate (Markgraf et al., 1993). However, as photoactivation usually occurs through the skull, resulting damage is only cortical, and reperfusion is not possible. Furthermore, photothrombosis does not produce a penumbral region resembling that which is observed in clinical stroke (Carmichael, 2005), potentially affecting the cellular processes that follow, thereby limiting the translational value of this model.

1.6.3 Middle cerebral artery occlusion

As previously highlighted, the MCA is often affected in ischemic stroke (Madden, 2002), hence the development of the most commonly used animal stroke model: middle cerebral artery occlusion (MCAo) (Koizumi et al., 1986). The MCA can be occluded via the internal (Boyko et al., 2010) or external (Longa et al., 1989) carotid artery, which are accessed at a ventral midline incision point. Occlusion can be temporary (Belayev et al., 1996) or permanent (Pena-Tapia et al., 2004). Transient MCAo is usually achieved by temporary insertion of a filament into the MCA. The filament is later removed, resulting in restoration of blood flow. Permanent MCAo involves leaving the filament in the MCA, or using a clip or cauterization to permanently

occlude blood flow. Occlusion results in infarction of both cortical and striatal regions, resembling pathology commonly observed following human stroke (Cramer, 2003).

However, there are several aspects to this stroke model that detract from its clinical relevance. Because of the vast territory supplied by the MCA, inadvertent damage often occurs (Lipsanen and Jolkkonen, 2011; Trueman et al., 2011), resulting in unintended behavioural impairments (Gerriets et al., 2004). Furthermore, while rats can often survive the large infarcts that are produced using MCAo, humans are unlikely to survive an injury of comparable extent (Carmichael, 2005). The acute nature of the occlusion, and if applicable the resolution, are also not representative of the corresponding clinical events, which are typically more progressive in nature (Kleim et al., 2007). Moreover, unless alterations are made to traditional MCAo procedures (Boyko et al., 2010), complications such as loss of blood supply (or direct damage) to the muscles of the mouth (Dittmar et al., 2005) can result in misinterpretation of post-surgical morbidity and/or the appearance of behavioural outcomes that result from surgical side effects rather than ischemia (Trueman et al., 2011). The procedure also carries a considerable risk of hemmorhage (Braeuninger and Kleinschnitz, 2009; Lipsanen and Jolkkonen, 2011), and even subtle variations in surgical technique can impact outcome and contribute to the variability inherent in the model (Trueman et al., 2011).

1.6.4 Endothelin-1

Endothelin-1 is a 21-amino acid peptide that acts through specific receptors to cause potent and long-lasting vasoconstriction. First described in 1988, there are three

forms of endothelin, designated ET-1, ET-2, and ET-3 (Inoue et al., 1989; Shah, 2007). The most potent vasoconstrictor, ET-1, is produced mainly by endothelial cells, but can also be produced by neuronal and glial cells throughout the CNS (Kuwaki et al., 1997; Naidoo et al., 2004; Nakagomi et al., 2000).

Endothelins act on one of two G-protein coupled receptors, designated ETR_A and ETR_B, that are predominantly expressed on smooth muscle cells (Naidoo et al., 2004; Shah, 2007). Precursor protein 'big ET-1' is converted to ET-1 by endothelin converting enzyme (Shah, 2007). ET-1 binds irreversibly to ETR_A (Rubanyi and Polokoff, 1994), resulting in increased intracellular Ca^{2+} and rapid and sustained vasoconstriction (Bourque et al., 2011; Shah, 2007); the precise mechanism of this process is not known (Kuwaki et al., 1997). Normal tension can be restored upon eventual endocytosis of the ET-receptor complex (Shah, 2007). The role of ETR_B remains controversial, because selective activation of the receptor can induce either vasodilation or constriction (Chuquet et al., 2002).

Because of its potent vasoconstricting properties, ET-1 can be used to induce localized ischemia when applied topically (Adkins et al., 2008; Fuxe et al., 1992), injected intracerebrally (Windle et al., 2006), or injected proximal to the MCA (Sharkey et al., 1993). ET-1 affects immediately proximal arteries, causing a rapid and temporary reduction in blood flow. The occlusion begins within 5-20 minutes (Fuxe et al., 1997; Macrae et al., 1993) and can last several hours (Macrae, 1992). The somewhat slower onset of ischemia resulting from ET-1 administration may be more representative of the clinical condition in stroke compared to the acute occlusion and reperfusion associated with MCAo (Kleim et al., 2007). A further advantage of this model is the greater degree of control over the infarct volume produced, afforded by adjusting the concentration,

volume, and stereotactic placement of the microinjections. Therefore, infarcts achieved using this model can be more localized and uniform than those resulting from traditional MCAo, which often produces large infarcts of questionable clinical relevance.

ET-1 has been used to produce ischemic injury to forelimb sensorimotor brain regions in both mouse (Horie et al., 2008) and rat (Adkins et al., 2004; Allred and Jones, 2004; Frost et al., 2006; Gilmour et al., 2004; Hughes et al., 2003; Soleman et al., 2010; Windle and Corbett, 2005; Windle et al., 2006) models. Windle et al. (2006) demonstrated that clinically relevant lesions could be produced by combining intracortical and intrastriatal injections of ET-1 (Figure 1.6). Resulting behavioural deficits have been shown to last from one week to more than a month (Adkins et al., 2004; Allred and Jones, 2004; Frost et al., 2006; Gilmour et al., 2004; Soleman et al., 2010; Windle and Corbett, 2005; Windle et al., 2006). A more detailed discussion of behavioural tests used to measure forelimb function will follow in Section 1.8.

While other stroke models discussed in this Section can result in similar behavioural outcomes, the ET-1 model was chosen for the research described in this thesis. The use of ET-1 offers 'face validity' and increases the likelihood of producing neuroplastic responses similar to those associated with clinical stroke (Gilmour et al., 2004). This permits the examination of the dynamic relationship between functional recovery and neuroplastic processes. Specifically, intracerebral (cortical + striatal) injections allow for control of lesion placement to target discrete functional areas while retaining sufficient intact neural tissue to act as a substrate for the putative cellular changes responsible for neuroplasticity.

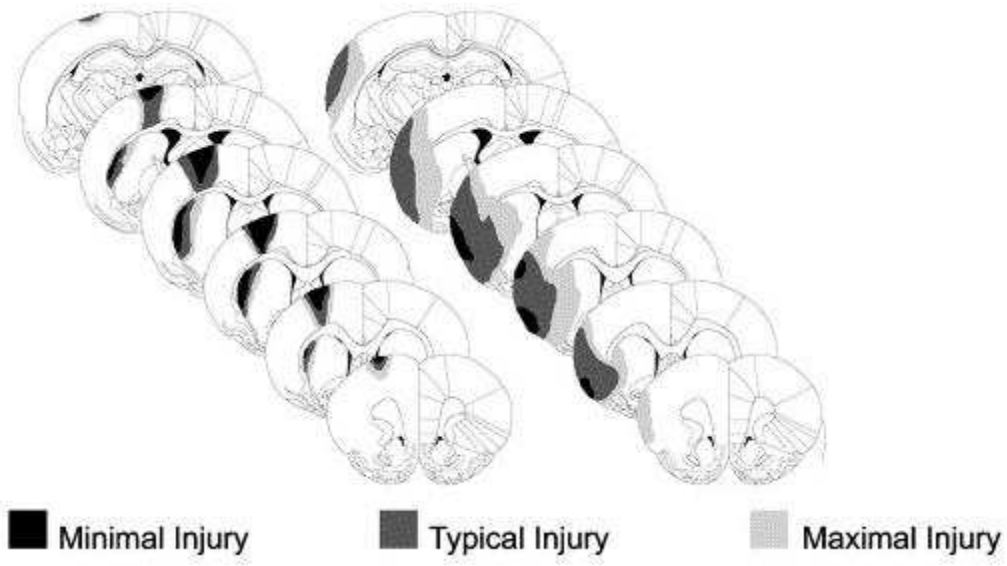


Figure 1.6. Ischemic damage caused by endothelin-1. Typical damage associated with select ET-1 model variations, manifested in coronal sections from approximately +3.8 to -2.0 (mm A/P) from bregma. The present study targeted cortical and striatal damage, as depicted in the first set of sections, as these areas are commonly damaged following clinical stroke [adapted from (Windle et al., 2006)]. Another variation of the ET-1 model is MCAo-proximal administration, which results in larger, more variable, and less forelimb-specific damage (second set of sections).

1.7 INFLUENCING NEUROPLASTICITY WITH REHABILITATION

The physical and emotional consequences of stroke can take an immense toll on patients and caretakers, while the economic burden of caring for patients represents a significant strain on health care budgets. Thus, basic and clinical scientists have long sought effective treatments to restore function following stroke. The discovery that a number of processes are involved in post-injury neuroplasticity, and that behavioural experience can be a potent modulator of these processes, has led to a significant shift in the field of rehabilitation therapy. However, activity-dependent changes can also be maladaptive. Compensation [the use of alternative behavioural strategies to perform a task (Whishaw, 2000)], as well as disuse (the absence of any post-injury activity) can lead to detrimental cellular changes in the brain (Benowitz and Carmichael, 2010). As such, rehabilitation aims to optimize behavioural experiences and influence neuroplasticity in a positive way, with the goal of normalization of function rather than compensation.

1.7.1 Constraint Induced Movement Therapy (CIMT)

One of the rehabilitative techniques that has been developed to increase use and improve function of the upper limb in survivors of stroke is CIMT (Nijland et al., 2011). The therapy discourages 'learned non-use', first described by Taub et al. (2006a). Learned non-use is a phenomenon whereby movement is initially suppressed due to failure and other adverse consequences encountered when a subject attempts to use the affected limb. This results in persistent compensatory behaviours and subsequent

suppression of use of the impaired limb, perpetuating the negative consequences (Figure 1.7). Through constraint of the unaffected limb and subsequent forced use of the affected one, CIMT encourages positive feedback about the limb's functional potential. Generally, a constraint device (e.g. a large mitten or sling) is worn for up to 90% of waking hours during a two week period (Richards et al., 2006; Sawaki et al., 2008; Wittenberg et al., 2003; Wolf et al., 2011), and is accompanied by intensive repetitive task practice (RTP) performed daily using the impaired limb. During RTP, patients engage in meaningful and increasingly difficult functional activities for which they receive positive feedback. In addition, other behavioural techniques such as home practice and problem solving sessions are used to aid in the transfer of functional gains to the performance of daily activities (Morris et al., 2006).

CIMT has been shown in the clinic to improve functional outcome (Bonaiuti et al., 2007; Nijland et al., 2011; Sawaki et al., 2008; Wittenberg et al., 2003; Wolf et al., 2011), even when administered to patients with chronic deficits (Liepert et al., 2000). However, the therapy presents several obstacles. CIMT is expensive, due the intensive therapy presently requiring specialized professionals. Furthermore, despite the fact that subjects are required to sign a 'behavioural contract' designed to increase incentive and ensure motivation (Taub and Uswatte, 2003; Wolf et al., 2011), CIMT is a demanding therapy for stroke patients who have diminished physical capacity. This raises concerns about compliance (Page et al., 2002), and several modifications of the therapy have been investigated in an attempt to reduce the prescribed intensity (Page et al., 2005; Sterr et al., 2002; Wolf et al., 2011). While results of these studies are promising, further confirmatory research is required. CIMT has only been developed for, and tested in, patients that retain some level of function, according to general inclusion/exclusion

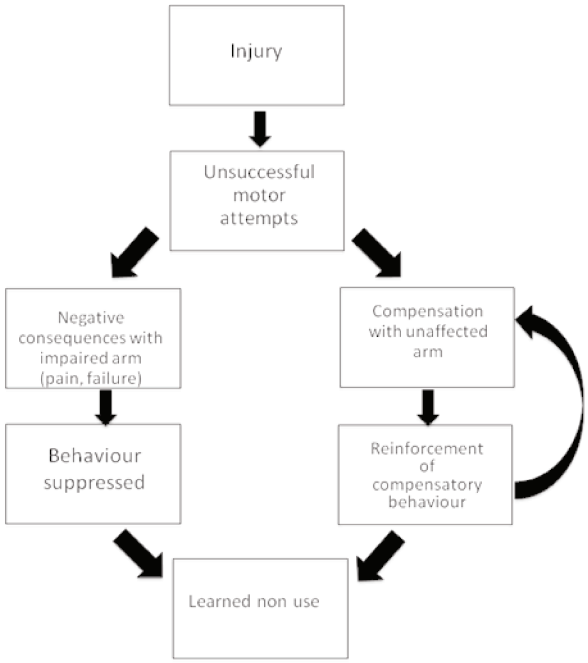


Figure 1.7. Principle of learned non-use following deficit in arm function. Following injury to the forelimb, attempts to use the impaired limb lead to unsuccessful outcomes. Consequences may include pain and failure, creating negative feedback which leads to suppression of the behaviour. Meanwhile, compensation with the unaffected or less affected limb results in higher success, reinforcing the behaviour. Together, these constitute the phenomenon of learned non-use. Adapted from (Taub et al., 2006a).

criteria such as the ability to initiate finger movements (Wolf et al., 2011), and extension movement of at least 20 degrees in the wrist and fingers (Richards et al., 2006; Sawaki et al., 2008; Taub et al., 2006b). This limits the generalization of study results to more disabled patients (Mark and Taub, 2004; Sterr and Saunders, 2006).

Unfortunately, clinical evaluation of CIMT is limited by the inability to control experimental variables. Size, location, and severity of the infarct, as well as co-morbidities and prior health status are all important variables that can affect treatment outcome (Duncan et al., 1992). There are also obvious challenges with respect to studying changes on a tissue and cellular level in the human stroke survivor population. Thus, clinical investigation into rehabilitation-driven neuroplasticity is challenging, highlighting the importance of developing valid, clinically relevant animal models of forced use rehabilitation.

1.7.2 Animal models of forced use rehabilitation

Valid experimental models are essential for the development of rehabilitative therapies to treat survivors of stroke. By modelling CIMT in animals, it may be possible to elucidate underlying processes responsible for functional benefits, and eventually to manipulate these processes to accelerate or enhance post-stroke recovery. Indeed, there have been several attempts at the development of animal models of rehabilitation that resemble CIMT (described below and summarized in Table 1.2) (Bland et al., 2000; DeBow et al., 2003; Humm et al., 1998; Leasure and Schallert, 2004; Müller et al., 2008; Schabitz et al., 2004). These have provided important insight into the development of rehabilitation paradigms that best model particular and

Rehab Model	Advantages	Disadvantages	References
Constraint	-Most direct model of CIMT; -May allow constraint for specific durations, thereby allowing more complete evaluation -Conducive to studies of unilateral forced use	-Constraint devices may be stressful, confounding results; -Lack of behavioural pressure to use paretic arm despite constraint	(DeBow et al., 2003; Leasure and Grider, 2010; Müller et al., 2008; Schabitz et al., 2004)
Forcing use with locomotion	-Stimulates use of the paretic limb in a less aversive paradigm	-Can be stressful (involuntary forced use) or lack control over intensity (voluntary forced use); -Involves bilateral forced use	(Auriat et al., 2006; DeBow et al., 2003; Kim et al., 2005; Maldonado et al., 2008; Marin et al., 2003; Ploughman et al., 2009; Yang et al., 2003)
Encouraging use with environmental enrichment	-Stimulates use of the paretic limb in a less aversive paradigm	-Complicated by other, non-forced use therapy components such as cognitive stimulation; -Involves bilateral forced use	(Auriat and Colbourne, 2009; Auriat et al., 2010; Biernaskie and Corbett, 2001; Biernaskie et al., 2004; MacLellan et al., 2011)
Task specific exercises	-An addition to rehabilitation that models task specific shaping exercises of CIMT	-Requires the desire of animals to participate in a demanding task	(Auriat and Colbourne, 2009; Auriat et al., 2010; Biernaskie and Corbett, 2001; Biernaskie et al., 2004; DeBow et al., 2003; Maldonado et al., 2008; Ploughman et al., 2009)

Table 1.2. Common animal models of forced use rehabilitation. A summary of advantages and disadvantages of several animal models of forced use rehabilitation.

appropriate aspects of the regimen. CIMT involves constraint of the intact limb for most waking hours, which forces use of the impaired limb for daily tasks as well as intensive therapist-led RTP exercises (see Section 1.7.1). In rats, this presents a challenge. The affected forelimb can be constrained (DeBow et al., 2003; Leasure and Schallert, 2004; Schabitz et al., 2004), but increased animal stress (Ke et al., 2011; Yanagita et al., 2007) and lack of behavioural pressure to rely on the impaired forelimb (DeBow et al., 2003) can be problematic. Some researchers have opted to shift focus away from constraint per se and toward forced use. Many also incorporate task-specific exercises to model the RTP associated with CIMT. However, this results in a 'trade off' of aspects of the rehabilitation, in an attempt to replicate specific components of therapy. For example, experimental rehabilitation may require implementing less intense but voluntary paradigms to reduce animal stress and increase behavioural incentive. Taub and Uswatte (2003) have emphasized that the 'constraint' used in CIMT is intended as a means to induce patients to use the affected extremity for a large proportion of the time and for a variety of activities, thus other strategies that induce similar pressures are likely to result in similar use-dependent reorganization and functional benefit (Taub and Uswatte, 2003).

1.7.2.1 Forcing use by constraint

The principle behind this approach is straightforward, and involves direct constraint of the unaffected forelimb, which forces use of the impaired forelimb. This strategy can be achieved by permanent plaster casting (Bland et al., 2000; DeBow et al., 2004; Humm et al., 1998; Kozlowski et al., 1996; Leasure and Schallert, 2004; Müller et

al., 2008; Schabitz et al., 2004), bandaging (DeBow et al., 2004), or use of a constraint jacket (DeBow et al., 2003).

Plaster casting and bandage wrapping have been used to model immediate and permanent constraint of the intact forelimb following electrolysis (Humm et al., 1998; Kozlowski et al., 1996), artery occlusion (Bland et al., 2000, 2001), photothrombosis (Müller et al., 2008; Schabitz et al., 2004) and devascularisation (DeBow et al., 2004). However, rather than leading to improved functional recovery, a number of studies have reported that this form of rehabilitation in rats results in use-dependent exaggeration of infarct volume (DeBow et al., 2004; Humm et al., 1998; Kozlowski et al., 1996) and either no functional improvement (Schabitz et al., 2004) or worsening of behavioural deficits (Bland et al., 2000, 2001; DeBow et al., 2004; Humm et al., 1998; Kozlowski et al., 1996; Müller et al., 2008). A possible explanation for these findings is the potential stress associated with complete constraint. Stress can decrease phosphorylation of CREB and inhibit BDNF production (Schaaf et al., 1998; Ueyama et al., 1997), thereby potentially attenuating plasticity (see Figure 1.4). Other explanations for the exacerbation of damage may involve excitotoxicity (Humm et al., 1999) and hyperthermia (DeBow et al., 2004) in perilesional tissue.

Debow et al. (DeBow et al., 2003) employed a sleeveless constraint jacket, which was worn around the upper torso of the rat and could be attached to a separate metal wrist bracelet to restrict forelimb movement. An advantage of this paradigm over plaster casting is the ability to control the duration of the constraint. This more closely resembles the clinical administration of CIMT. However, the authors reported that constraint (for 6h/day) using this paradigm did not produce functional benefit. As above, a possible explanation of the failure of this seemingly clinically relevant model of CIMT

is that the continuous wearing of the jacket, or the constraint period used, could exacerbate animal stress and attenuate neuroplastic processes. A further explanation for the absence of improvement using this model may be the lack of behavioural pressure to use the impaired forelimb. In the same study, when animals were further engaged by performing additional rehabilitative exercises with the damaged limb (resembling RTP), functional recovery was effectively promoted (DeBow et al., 2003).

1.7.2.2 Encouraging use with activity: enriched environment

In 1947, Canadian neuroscientist Donald Hebb noted that laboratory rats that had been taken home as pets had better learning and problem-solving skills that those who lived in standard laboratory conditions (Hebb, 1947), suggesting that an enriched environment (EE) may affect neuroplasticity. Since then, research into exposure to EEs has shown that EEs are effective at improving post-stroke functional recovery (Auriat and Colbourne, 2009; Auriat et al., 2010; Biernaskie and Corbett, 2001; Janssen et al., 2010; MacLellan et al., 2011), in a time-dependent manner (Biernaskie et al., 2004).

Exposure of rats to enriched environments provides ample opportunity for engagement in voluntary nonspecific activity, because a major component of these environments is the presence of novel objects and climbing apparatuses. As such, general voluntary movement is encouraged in a way that stimulates use of the impaired forelimb, which may contribute to activity-dependent plasticity. While the precise contribution of the motor enrichment component of EE to functional recovery has yet to be determined, this environment represents a basic voluntary use paradigm that has shown significant positive results (Hicks et al., 2007; Janssen et al., 2010).

1.7.2.3 Forcing use by locomotion

Locomotion presents an alternative approach to encourage use-dependent plasticity, by exploiting the fact that animals need to engage the impaired forelimb in order to run or walk. This form of rehabilitation can be forced, using motorized running wheels or treadmills (Auriat et al., 2006; Kim et al., 2005; Ploughman et al., 2009; Yang et al., 2003), or voluntary, by allowing free access to running wheels (Maldonado et al., 2008; Marin et al., 2003). The intensity of such therapies has varied from < 1km to 7 kms per day, and lasted 3-5 weeks (Auriat et al., 2006; DeBow et al., 2003; Kim et al., 2005; Maldonado et al., 2008; Ploughman et al., 2009; Yang et al., 2003).

The use of locomotion appears to be a promising approach, and both forced (Kim et al., 2005; Leasure and Grider, 2010; Ploughman et al., 2009; Yang et al., 2003) and voluntary (Ke et al., 2011; Mizutani et al., 2011) locomotion can improve functional recovery following administration of ET-1 topically (Leasure and Grider, 2010), directly proximal to the MCA (Ploughman et al., 2009), and following MCAo using the intraluminal thread method (Ke et al., 2011; Kim et al., 2005; Mizutani et al., 2011; Yang et al., 2003). Additionally, unlike direct constraint methods described above, infarct volume is not affected (Leasure and Grider, 2010; Ploughman et al., 2009) or is even decreased in animals receiving locomotor rehabilitation (Yang et al., 2003). The mechanisms underlying functional recovery in these studies may involve increased expression of BDNF (Ke et al., 2011; Kim et al., 2005), because blocking BDNF production attenuates the functional recovery induced by exercise (Ploughman et al., 2009). Conversely, several groups have reported that voluntary (Maldonado et al., 2008; Marin et al., 2003) and forced (Auriat et al., 2006) exercise exerts no beneficial effect on

functional recovery (although, notably, without exacerbating damage). The reason for this discrepancy could be variations in the paradigms of both forced and voluntary exercise that have been studied, and the use of different outcome measures.

Ke et al. (2011) showed that voluntary exercise (access to voluntary wheel running for 23 hours per day) is more beneficial than forced exercise (30 minutes of daily treadmill exercise). This study demonstrated that forced exercise resulted in increased levels of corticosterone, a typical marker of stress, which can inhibit BDNF expression (Schaaf et al., 1998). Similar results were reported by Yanagita et al. (2007) who demonstrated that following voluntary wheel running, animals exhibited fewer neurons containing corticotrophin-releasing hormone compared to forced treadmill exercise controls. However, a drawback to voluntary exercise is that the experimenter has less control over the intensity of the rehabilitation (Ke et al., 2011; Maldonado et al., 2008; Mizutani et al., 2011).

1.7.2.4 Forcing use with task specific exercises

Clinical CIMT includes RTP exercises that involve grasping, gripping, and manipulating objects (Sawaki et al., 2008; Wittenberg et al., 2003). Therefore, many preclinical researchers combine one of the forms of rehabilitation described above with a task specific exercise (Auriat et al., 2010; Biernaskie and Corbett, 2001; Biernaskie et al., 2004; DeBow et al., 2003; Fang et al., 2010; Maclellan et al., 2005; Maldonado et al., 2008; Ploughman et al., 2009). Experimentally, RTP exercises are often modelled using pellet reaching tasks. Animals are provided palatable sucrose pellets that require reaching with the impaired limb. This can be achieved by placing pellets on the

appropriate side of a tray, shelf, or well (Auriat et al., 2010; Fang et al., 2010; Maldonado et al., 2008) or by using a modified staircase apparatus (Auriat and Colbourne, 2009; Biernaskie and Corbett, 2001; Biernaskie et al., 2004).

1.7.2.5 Initiating rehabilitation: considerations

While CIMT is generally initiated as soon as the patient is medically stable, it is still effective when initiated several years post-insult (Liepert et al., 2000). Some animal studies have shown that initiation of rehabilitation immediately after surgery can result in larger infarcts and worse functional outcome (DeBow et al., 2004; Humm et al., 1998). However, unlike humans, rats exhibit spontaneous recovery on many functional tests in a relatively short time frame (< 1 month), so delayed rehabilitation may result in a missed opportunity to accelerate recovery. Thus, rehabilitation in rat models is generally initiated between post surgical day 3 and 7 (Auriat et al., 2010; Biernaskie et al., 2004; DeBow et al., 2003; MacLellan et al., 2011; Maldonado et al., 2008; Ploughman et al., 2009). Considering that post-ischemic neuroplastic processes are dynamic and may depend on the severity of the initial insult, the ideal time to initiate a particular rehabilitation model likely varies.

1.8 MEASURING FUNCTIONAL DEFICIT AND RECOVERY

When evaluating post-stroke deficits and subsequent recovery in animal models, it is recommended that a battery of tests be used (Kleim et al., 2007; Schaar et al., 2010; STAIR, 1999). Several considerations that must be made when choosing behavioural

tests include the sensitivity of the test to the damage being observed, the time required to pre-train and test animals, and the possible confounding properties of the tests and testing schedule chosen. The latter is especially important in studies of rehabilitation, in which performance of the tests themselves can affect activity-dependent neuroplasticity, and therefore, be potentially rehabilitative (Kleim et al., 2007).

An ongoing challenge for both clinical and experimental researchers is how to appropriately define and assess 'recovery'. In order to measure function, researchers rely on a variety of different tests and scales. Sometimes, what appears to be 'recovery' is simply the subject (human or animal) compensating for lost function in a way that allows them to attain a performance on the test that resembles normal function. This is an important distinction; if using a scoring system such as a scale that measures competency on activities of daily living, a patient who has learned to carry out daily living tasks using their other limb would show improved scoring despite lack of any actual recovery. Incorporation of a variety of functional tests decreases the chance of compensatory action to confound the result.

Several behavioural tests of forelimb function have been well characterized in rat stroke models, and the tests chosen for the present research are summarized in Table 1.3. These tests range from those that measure acquired sensorimotor behaviours (i.e. skilled movements, which generally require training) to those that measure pre-existing behaviours (i.e. unskilled movements and reflexes requiring very little training). Detailed descriptions of the methodological details are presented in Chapters 2, 3, and 4 (see Sections 2.2.4, 3.2.4, and 4.2.4).

Test	Description	Evaluating behaviour	Selected references
Forelimb flexion	The rat is suspended above the home cage and the position of the impaired forelimb is scored from 0 to 2 wherein 0 = no flexion, 1 = flexion of the forelimb, and 2 = forelimb flexion with twisting of the torso.	Basic neurological function	(Bederson et al., 1986; Riek-Burchardt et al., 2004; Sun et al., 2008)
Forelimb placing	The rat is held by the torso with all but the testing limb secured. The vibrissae or wrist of the animal is brushed against the edge of a table. The number of successful responses (placing the paw in response to the simulation) is recorded.	Sensorimotor capacity	(Leasure and Schallert, 2004; De Ryck et al., 1989; Schallert et al., 2000a)
Cylinder	The animal is placed into a transparent plastic cylinder which encourages vertical exploration. He is then video recorded while rearing and exploring. The percent usage of the impaired forelimb is determined by slow motion video analysis.	Preference for intact limb for postural support during movements	(Auriat and Colbourne, 2009; Hicks et al., 2009; MacLellan et al., 2011; Schallert et al., 2000a; Soleman et al., 2010; Windle et al., 2006)
Horizontal ladder	Animals cross a horizontal ladder of variably spaced rungs to escape aversive stimuli (noise and light). The average number of foot slips made is determined by video analysis.	Coordination and sensorimotor function	(Auriat et al., 2006, 2010; Biernaskie et al., 2004; DeBow et al., 2003; Metz and Whishaw, 2002; Soleman et al., 2010)
Staircase	Animals are placed into an apparatus consisting of an elevated platform with descending steps on either side. Each step is baited with multiple palatable sucrose pellets, at progressively deeper levels. Dexterous movements requiring motor function and sensory feedback are required to obtain pellets.	Assesses forelimb sensorimotor capacity, dexterity, and coordination	(Auriat and Colbourne, 2009; Biernaskie et al., 2004; DeBow et al., 2003; MacLellan et al., 2011; Montoya et al., 1991)

Table 1.3. Behavioural tests of animal forelimb function. A summary of the behavioural tests used in this thesis. Methods are described in more detail in Chapters 2, 3, and 4, under Methods and Materials.

1.9 SUMMARY

With an aging population, high incidence of stroke, and few effective medical interventions, it is vital to gain a better understanding of the mechanisms underlying functional recovery in order to refine existing rehabilitative therapies and to develop new techniques. In order to understand the mechanisms underlying rehabilitation, clinically relevant animal models are important. Addressing all aspects of human stroke using any single animal model may never be possible, but many animal models have contributed to our understanding of the mechanisms underlying ischemic and neuroplastic processes.

Modelling rehabilitation presents a major challenge and remains fundamentally different from rehabilitation in a clinical setting. Stroke patients undergoing CIMT receive supervised, assisted, and highly-motivated rehabilitation with trained experts. Experimental rehabilitation is largely hands-off, and animals are more difficult to motivate. The validity of any model may vary with respect to a particular aspect of the therapy. However, cortical reorganization and functional recovery may share underlying processes with the mechanisms responsible for the benefits of CIMT. In the clinic, constraint is simply considered the tool that induces the beneficial use of the impaired limb, discourages learned non-use, and encourages beneficial neuroplastic processes to occur (Taub and Uswatte, 2003).

1.10 EXPERIMENTAL OBJECTIVES OF THE THESIS

The research described in this thesis was performed to investigate post-ischemic functional recovery and neuroplasticity in response to a novel model of voluntary forced use of the impaired forelimb. It was hypothesized that **a novel model of voluntary forced use movement therapy would result in improved functional recovery as measured using a battery of behavioural tests.** It was further hypothesized that this rehabilitation would **alter the expression of a number of markers of neuroplasticity.** These hypotheses were tested by addressing the following specific aims:

1. **Refine an endothelin-1 model of focal ischemic stroke in the rat, in order to obtain reproducible focal lesions to forelimb motor representation areas of the brain.**

   ET-1 was injected into the brain at a set of previously unpublished stereotaxic coordinates corresponding to the forelimb motor cortex and striatum. The animals were assessed on a number of behavioural tests to determine the magnitude and duration of deficit. Tissue was analysed to determine the placement, spread, and volume of the resulting lesion.

2. **Conduct a preliminary evaluation of a novel model of voluntary forced use movement therapy on functional recovery and markers of neuroplasticity.**

   Animals were treated with a novel rehabilitation model wherein they were allowed to voluntarily engage in the movement of pet activity balls, which forced use of the impaired forelimb using an appetitively motivated approach. They were again assessed on a number of behavioural tests (those

used in specific aim 1, as well as an additional test) to determine whether there was a benefit of rehabilitation on recovery profile. Tissue was analysed to determine whether rehabilitation affected the infarct volume, and whether there was any effect on several markers of neuroplasticity: BDNF, $NOGO_A$, and the presence of nascent neurons.

3. **Evaluate a refined model of voluntary forced use movement therapy based on functional measures and markers of neuroplasticity.**

   Animals were treated with a refined rehabilitation model wherein they were subjected to both activity ball therapy as well as a task specific exercise intended to model RTP that accompanies CIMT. Functional recovery was assessed using a number of behavioural tests (some of which were used in the previous specific aims, as well as an additional test). Tissue was analysed for infarct volume, presence of nascent neurons, and the level and cellular origin of BDNF.

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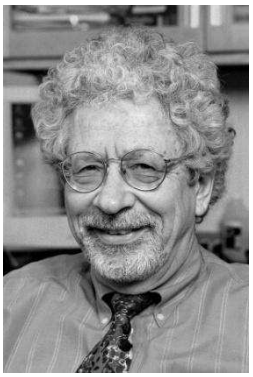
CHAPTER 2:

DEVELOPMENT OF THE ENDOTHELIN-1 MODEL OF POST-ISCHEMIC UPPER EXTREMITY IMPAIRMENT[1]



"The brain can reorganize to compensate for loss; it is enormously plastic."

-Paul Bach y Rita (1934-2006)
picture from http://just-one-question.com/Site/joq_pers_paul.html

[1] The majority of data presented in this chapter are published in: A novel approach to induction and rehabilitation of deficits in forelimb function in a rat model of ischemic stroke. Acta Pharmacol Sin. 2012, **34**(1): 104-112. JLT contributed to experimental design, performed surgeries, behavioural testing, and histology, and prepared the manuscript.

SUMMARY

Stroke commonly results from impaired circulation through the MCA, which supplies blood to sensorimotor regions in the striatum and cortex. Research into rehabilitation of motor function necessitates the use of an appropriate stroke model. Because recovery of function is largely attributed to numerous neuroplastic changes on a cellular level, it is especially important to ensure sufficient unaffected or salvageable tissue remains to act as a substrate for recovery processes. One surgical stroke model used in rodents is intracerebral injection of a vasoconstrictor, ET-1. By constricting blood vessels in surrounding tissue, ET-1 models the events of a temporary ischemic episode in a clinically relevant manner. Some research groups have published variations of the ET-1 model to produce localized ischemic damage, however no surgical standard exists for creating lesions specific to forelimb motor areas of the brain. In the study presented in this Chapter, adult male rats were randomly assigned to receive ET-1 microinjections at a series of previously unreported stereotaxic coordinates, or sham surgery. Animals were then assessed over a period of 21 days using a battery of sensorimotor tests to determine deficit and subsequent spontaneous recovery. Following the study, animals were then euthanized and infarct volume was measured. This ET-1 protocol resulted in reproducible, well-defined lesions, accompanied by deficits that lasted up to 21 days post-surgery. This surgical protocol was therefore deemed to be appropriate to use for studying the effects of post-ischemic rehabilitation in the following Chapters of this thesis.

2.1 INTRODUCTION

Stroke commonly results from impaired circulation through the MCA (Madden, 2002) which supplies blood to motor regions in the striatum and cortex (Yamori et al., 1976). In humans, arm impairment is a particularly debilitating and pervasive functional deficit following stroke (Williams et al., 1999), and proper function of the arm and hand is important for achieving post-stroke independence (Burvill et al., 1997). While some spontaneous recovery of deficits occurs in the early post-stroke period (Krakauer, 2005), this is largely attributed to the resolution of secondary injury processes, and chronic deficits can remain for years in the absence of rehabilitative intervention (see Section 1.3). In order to investigate processes underlying post-ischemic neuroplasticity, and to properly evaluate models of rehabilitation, it is essential to begin with an appropriate stroke model.

While several animal models of stroke exist, rat models remain the most popular choice for researchers. This is largely due to the similarity between rat and human neurovasculature (Macrae, 1992; Yamori et al., 1976), the relatively low cost and ease of use (Durukan and Tatlisumak, 2007), the large number of validated functional tests (Kleim et al., 2007; Tamura et al., 1981), and the documented similarities between rat and human limb movements (Whishaw et al., 2002).

All experimental stroke models are associated with inherent variability in the lesion that is produced. Because recovery of function is largely attributed to numerous neuroplastic changes at a cellular level (Dobkin, 2004; Jones et al., 2009; Wieloch and Nikolich, 2006) (see Section 1.4), it is especially important to ensure that sufficient unaffected or salvageable tissue remains to act as a substrate for recovery processes.

Furthermore, consideration should be given to complications of the model that can have a direct impact on behavioural outcome. For example, poor overall health due to loss of body weight, infections, and collateral damage to non-targeted areas can all affect performance on functional outcome measures.

Occlusion of the MCA is presently one of the most commonly used models of stroke in the rat (Durukan and Tatlisumak, 2007; Kleim et al., 2007) (see Section 1.6.3). Variations on the model include permanent occlusion (using a clip or suture) (Sun et al., 2012) or temporary occlusion (the insertion and later removal of an intraluminal filament, allowing reperfusion) (Belayev et al., 1996). However, both methods often result in large, inconsistently placed lesions, rendering them inadequate for studying specifically localized deficits. Additionally, they are commonly associated with relatively high mortality (Lindner et al., 2003) unless accompanied by labour-intensive post-operative care (Sun et al., 2008). Furthermore, mechanical occlusion and reperfusion are instantaneous, unlike the more gradual processes that take place during clinical stroke (Kleim et al., 2007).

Alternative models of focal stroke have been developed in an attempt to more effectively control lesion location and reproducibility, and to produce marked deficits specific to forelimb function. Non-ischemic focal lesioning techniques such as devascularisation (Gonzalez and Kolb, 2003) and photothrombosis (Shanina et al., 2006) can result in precise and discrete lesions, however these affect surface tissue only (without accompanying damage to subcortical areas), do not allow for restoration of blood flow to the ischemic region, and may not result in the same post-ischemic processes as vessel occlusion.

An alternative method is to reversibly constrict local vessels pharmacologically, through administration of a vasoconstricting peptide, ET-1. Endothelin-1 causes a rapid, temporary, and dose-dependent constriction of affected arteries, resulting in interruption in blood flow within minutes, followed by reperfusion over several hours (Fuxe et al., 1997; Macrae, 1992; Nikolova et al., 2009). Depending on the brain regions being targeted, ET-1 can be applied to the MCA (Robinson et al., 1990; Sharkey et al., 1993), or to the cortical surface (Fuxe et al., 1997), injected intracerebrally (Gilmour et al., 2004), or administered as a combination of intracerebral and topical application (Soleman et al., 2010; Windle et al., 2006). Intracerebral microinjections, in which lesion placement can be carefully controlled and subcortical areas can be affected, represent a promising model for studying clinically relevant recovery and rehabilitation (see Figure 1.6). While variations on the model have been reported (Gilmour et al., 2004; Hewlett and Corbett, 2006; Windle et al., 2006), no surgical standard presently exists.

Previous work in our laboratory aimed to develop a variation of the ET-1 model that would result in reproducible unilateral ischemic lesions to the forelimb sensorimotor cortex and dorsolateral striatum, as well as functional forelimb impairments with an associated measurable recovery period (Hume, 2009). The specific aim of the current study was to further optimize the surgical technique using an injection protocol of four cortical injections (1µl each) and one striatal injection (0.5µl) of a 400 pmol/µl ET-1 solution.

2.2 METHODS

2.2.1 Experimental animals

Adult male Sprague-Dawley rats (N=20) were purchased from Charles River Laboratories (Montreal, Canada) and single housed on a 12 hr light/dark cycle (lights on at 08:00; off at 20:00). Animals had ad libitum access to food and water, and weighed between 300-350 g at the time of surgery. All procedures were conducted in accordance with the guidelines of the Canadian Council for Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee.

2.2.2 Endothelin-1 preparation

One day prior to surgeries, lyophilized ET-1 (Calbiochem, Germany) was dissolved in sterile H_2O at a concentration of 400 pmol/µl. The prepared ET-1 was then stored as aliquots at -20^{o}C until use, up to a maximum of 12 days.

2.2.3 Surgical procedure

Forelimb sensorimotor coordinates for ET-1 injections were determined using the Paxinos and Watson Rat Brain Atlas (Paxinos and Watson, 2007). Prior to the study, injection locations were verified using injections of cresyl violet dye into the chosen coordinates using a separate set of animals (n=2). Before surgery, rats were randomly allocated to the Sham or Stroke group. At the time of surgery, rats were placed into an

induction chamber filled with 3.5% isoflurane in oxygen for 8 minutes; anaesthesia was subsequently maintained during the surgery using 2% isoflurane in oxygen (or as required). Once anesthetised, animals were mounted onto a stereotaxic apparatus (David Kopf Instruments, USA). The scalp was shaved, topical anaesthetic (Xylocaine; AstraZeneca, Canada) was applied, a midline incision was made, and the scalp was retracted with clamps. The surface of the skull was cleared of all tissue, the skull was confirmed to be level between bregma and lambda, and injection coordinates were marked (Table 2.1). Small holes were drilled through the skull at each injection location using a stereotaxically mounted drill (Stoelting Co., USA). At each location, a 26 gauge 10 µl syringe was lowered into brain tissue and left undisturbed for 1 min. Endothelin-1 was then injected at a flow rate of 0.5µl/minute (injection volumes in Table 2.1), after which the needle was left undisturbed for 4 minutes before being slowly retracted from the brain. Following all injections, the scalp was sutured and the incision site was treated with topical anaesthetic. Body temperature was monitored regularly and maintained at 36.0 ± 0.2°C for the duration of surgery using a heating pad. Following surgery, animals were given subcutaneous injections of butorphanol (2.0 mg/kg), returned to their home cage, and allowed to recover. A heating pad remained under the home cage for 1 hour post-surgery. Sham-operated rats received the same surgical procedure up to but not including the drill holes, remained anesthetised for the same amount of time as the ET-1 animals, and received the same post-operative medication (n=14 stroke; n=6 sham).

	Stereotaxic coordinates (mm from bregma)			Volume (µl)
	AP	ML	DV	
Cortex injection 1	+2.5	-2.8	-2.5	1.0
Cortex injection 2	+1.6	-2.8	-2.5	1.0
Cortex injection 3	+0.9	-2.8	-2.5	1.0
Cortex injection 4	+0.4	-2.8	-2.5	1.0
Striatum injection	+0.9	-3.7	-6.0	0.5

Table 2.1 ET-1 injection coordinates. Endothelin-1 microinjection locations (in mm from bregma) used in this study. Coordinates determined using (Paxinos and Watson, 2007). AP = anterior/posterior; ML = medial/lateral; DV = dorsal/ventral. A visual representation of the injection coordinates approximately shows the cortical injections along the forelimb motor representation of the cortex (solid dots) and the deeper striatal injection location (star)

2.2.4 Behavioural testing

Several behavioural tests commonly employed to determine sensorimotor deficits were used in this study. For three weeks prior to surgery, animals were handled daily and acclimated to all tests, then pre-surgical scores were recorded. Following surgery, animals were tested regularly until the end of the study at post surgical day (PSD) 21. When evaluating behaviour, 'deficit' was defined as a mean post-surgical stroke group performance significantly worse than mean sham control performance and 'recovery' was considered the point at which the mean stroke group score was not significantly different from mean sham performance. In any instance where the stroke group had recovered at a particular time point, then had subsequent testing days with impaired performance, 'recovery' was considered the latest time point at which they consistently performed at sham level. All performance evaluations were conducted by an experimenter blind to the surgical condition.

2.2.4.1 Forelimb placing tests

Two tests of forelimb placing were employed daily post-surgery: tactile-stimulated forelimb placing (TFP) and vibrissae-stimulated forelimb placing (VFP). Rats were held by their torso, with 3 limbs secured and the forelimb to be tested hanging free. For TFP, the distal portion of the unrestrained forelimb was brushed against the edge of a table. For VFP, the rat was moved horizontally toward the table edge until the vibrissae came in contact with the surface (Figure 2.1). For each forelimb, the deficit

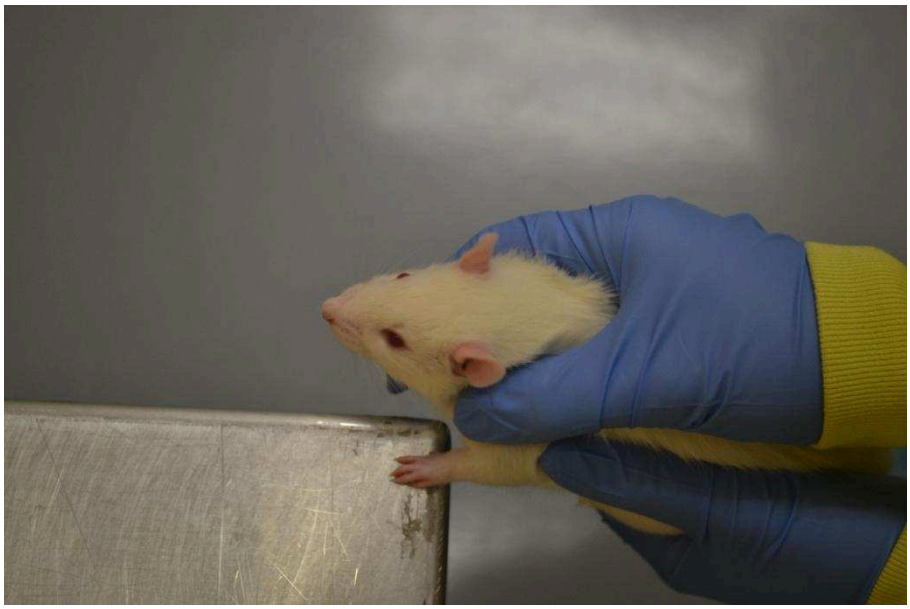


Figure 2.1. Forelimb placing tests. Vibrissae and tactile-stimulated forelimb placing testing was performed by constraining three limbs (except the forelimb to be tested) and gently brushing the vibrissae or wrist of the rat against a table edge. In response, the rat would place a forepaw onto the table when stimulated. Following stroke surgery, this response was disabled.

was determined based on the number of failed responsive forelimb placements on the table top out of 5 attempts. Attempts that missed the tabletop or did not place within 2 seconds of stimulation were considered unsuccessful. Testing took place during the light cycle, but under red light conditions in order to diminish the influence of visual input on placing success. The order of ipsilateral/contralateral forelimb testing was alternated.

2.2.4.2 Forelimb postural reflex test

The forelimb postural reflex test was performed daily. Rats were suspended by the base of the tail 50 cm above their home cage. The position of the contralesional forelimb was scored as follows: normal vertical reaching toward the surface (score = 0; no deficit), $>90^{o}$ forelimb extension at the shoulder or adduction of the forelimb to the torso (score = 1; mild-moderate deficit), or extension/adduction accompanied by torso twisting (score = 2; severe deficit).

2.2.4.3 Cylinder test

Animals were tested for limb preference and asymmetry in postural weight support during exploratory activity using the Schallert cylinder test (Schallert et al., 2000) on PSD 1, 3, 6, 10, 14, 18 and 21. Rats were placed inside a clear open-ended cylinder (25cm diameter x 30cm height) and allowed to rear and explore the cylinder walls during 15 rears (Figure 2.2). A camera was stationed above the top of the cylinder to record forepaw usage during exploratory behaviour. Videos were then analyzed to determine the number of forelimb wall contacts made. The percentage use of the

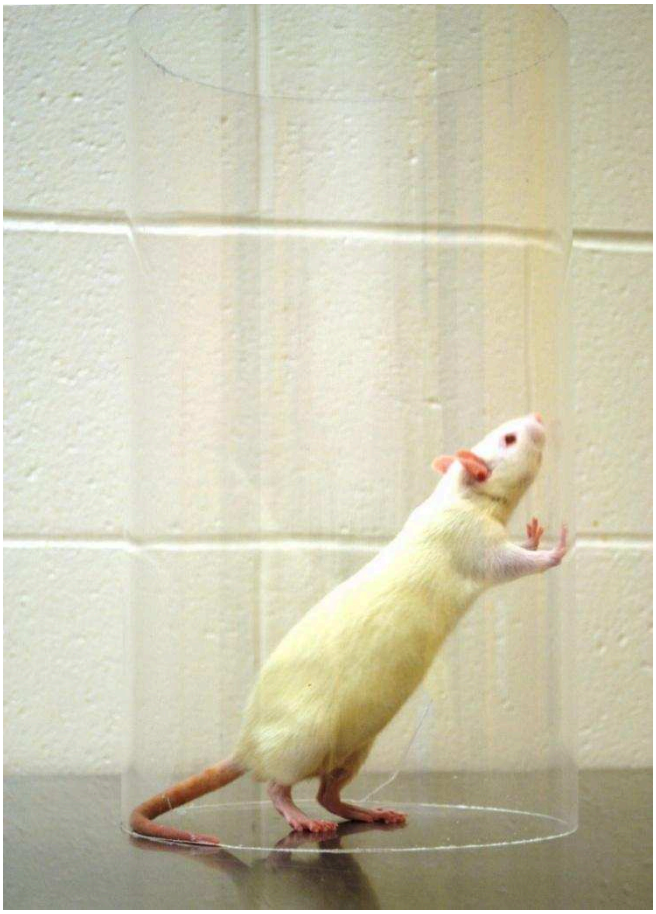


Figure 2.2. Cylinder test. Animals were placed into a clear plastic cylinder, and allowed to rear several times while being filmed from above. Intact animals would use each forelimb for approximately half of all exploratory and weight bearing movements, thus a post-injury measure of asymmetry can be obtained by testing animals following stroke surgery.

impaired forelimb was determined using the following formula: [(# contralesional touches + ½ # bilateral touches) / total # touches] *100.

2.2.5 Histology and infarct quantification

Following behavioural testing, rats were deeply anesthetised with sodium pentobarbital (240 ml/kg; Euthansol, Schering, Canada) and decapitated. Brains were quickly removed to 250 ml of 10% phosphate buffered neutral formalin for 72 hours, sectioned (150 µm) using a Vibratome 1000 Plus (Vibratome Co. St. Louis, USA), mounted on slides, and stained using 0.1% cresyl violet solution. Photographs (Canon EOS DS6041 camera) of every second section (i.e. 300 µm apart) were then used for quantifying infarct damage using Image J software (National Institutes of Health [NIH], USA) by overlaying each experimental section onto a corresponding template section, and tracing the area of injury. Ischemic injury was defined as pallor or lack of cresyl violet staining (Luke et al., 2004). The total volume of brain injury was determined by summing the area of damage from each section and multiplying that value by the distance between the measured sections (Swanson et al., 1990).

2.2.6 Statistical analyses

Statistical analyses were performed using PAWS Statistics (v. 18; SPSS Inc., USA). Behavioural data were analysed using repeated measures analysis of variance (RM ANOVA). Where the sphericity assumption was violated (Mauchly's test; $p<0.05$) the Greenhouse–Geisser correction was applied (Geisser and Greenhouse, 1958). This

alters the degrees of freedom such that the critical F-value remains unchanged while the Type 1 error that results from violating sphericity is decreased (degrees of freedom are reported to the nearest integer). Independent two-tailed t-tests were used to determine differences between groups on specific days. Significance was considered $p<0.05$. Values are expressed as mean ± SEM.

2.3 RESULTS

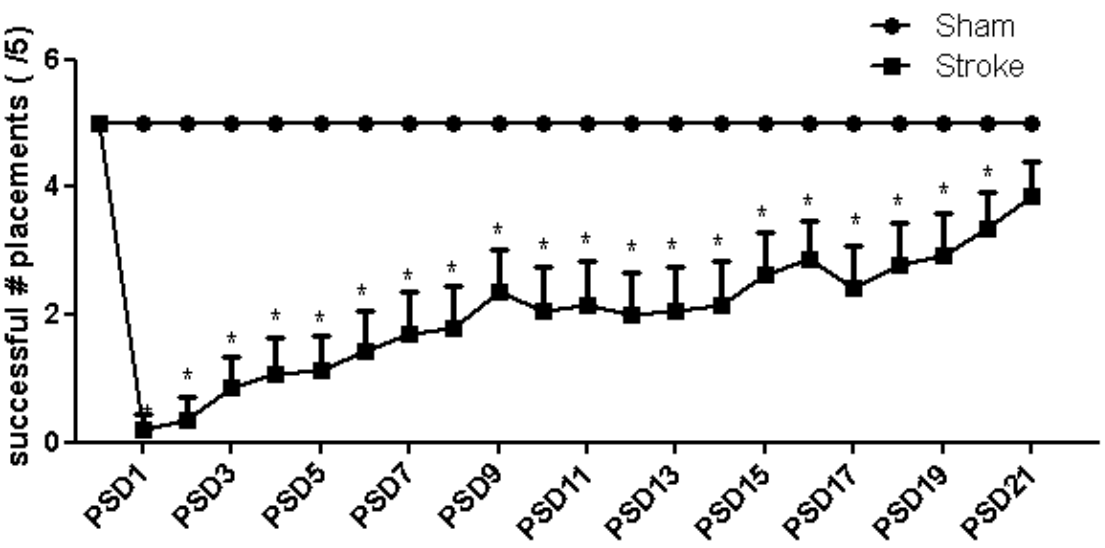
2.3.1 Behavioural tests

2.3.1.1 Forelimb placing tests

Prior to surgery, all animals were able to successfully place 5/5 times in response to both tactile and vibrissae stimulation. Following ET-1 surgery, the ipsilateral placing response remained intact (data not shown). For tactile-stimulated placing on the contralesional side, there was a significant effect of group ($F_{1,18}$=15.53; p=0.01), an effect of time, and a significant interaction of group x time ($F_{4,69}$=3.79; p=0.008). Stroke animals remained impaired until PSD 20 (p=0.013) (Figure 2.3A).

In the vibrissae-stimulated forelimb placing test, there was a significant group effect ($F_{1,18}$ =15.53; p=0.001), and a significant effect of time as well as interaction of group x time ($F_{4,68}$ =5.72; p=0.001). Stroke resulted in a significant impairment in contralateral forelimb placing in response to vibrissae stimulation that lasted for the duration of the study (p=0.030) (Figure 2.3B).

A.



B.

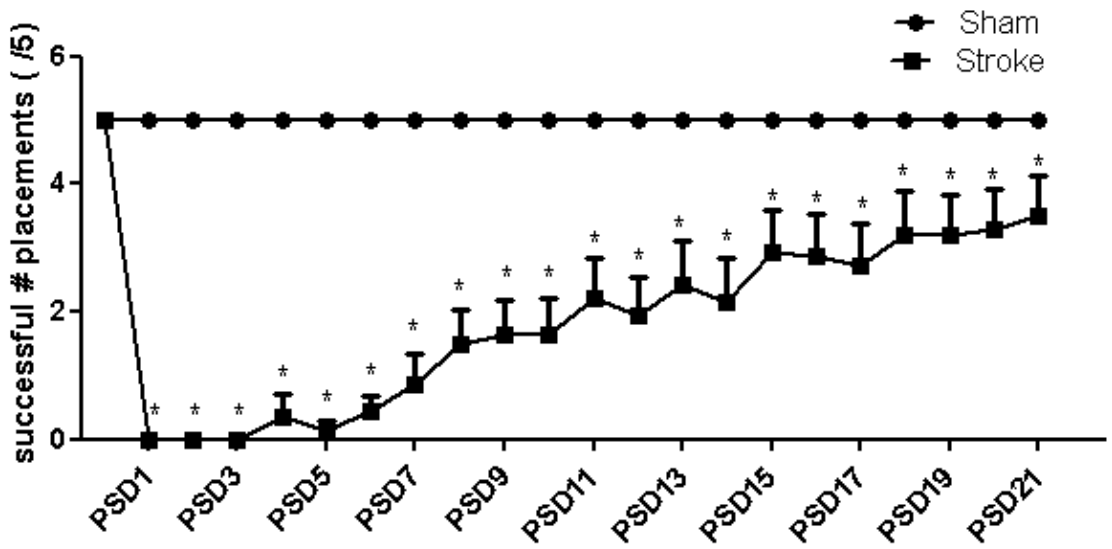


Figure 2.3. Performance on the forelimb placing tests. (A) Contralesional tactile-stimulated forelimb placing function was impaired following ET-1 surgery, but not Sham surgery. The deficit was significant until 20 days post surgery. (B) Vibrissae-stimulated forelimb placing function was impaired following ET-1 surgery but not Sham surgery. The deficit was significant until 21 days post surgery. n=14/stroke n=6/sham.

2.3.1.2 Forelimb postural reflex test

Prior to surgery, animals in both the Stroke and Sham groups all received a score of 0 in the forelimb postural reflex test. Following surgery, statistical analysis revealed a significant difference between groups ($F_{1,18}$=10.8; p=0.004), a significant effect of time, and an interaction of group x time ($F_{5,86}$= 3.26; p=0.011). Postural reflex remained impaired in ET-1 animals for the duration of the study (p= 0.002) (Figure 2.4).

2.3.1.3 Cylinder test

To assess differences in the spontaneous use of the contralateral forelimb during exploration, the cylinder test was used to evoke and analyze percent forelimb usage. Sham and Stroke groups performed similarly prior to surgery, relying on the contralesional forelimb for exploring and weight bearing approximately half of the time (51.5 ± 2.8% for Sham and 49.1 ± 3.8% for Stroke). Following surgery, animals that received ET-1 demonstrated a consistent tendency to rely less on their contralesional forelimb compared to shams (PSD 1 = 39.1 ± 4.7% vs 48.4 ± 2.6%), although there was no significant difference between groups ($F_{1,18}$=1.978; p=0.177) (Figure 2.5).

2.3.2 Histology and infarct quantification

A total of five intracerebral injections of ET-1 were administered in this study, targeting brain regions known to be responsible for forelimb sensorimotor function, including sensorimotor cortex and dorsolateral striatum (Figure 2.6A). Brains were

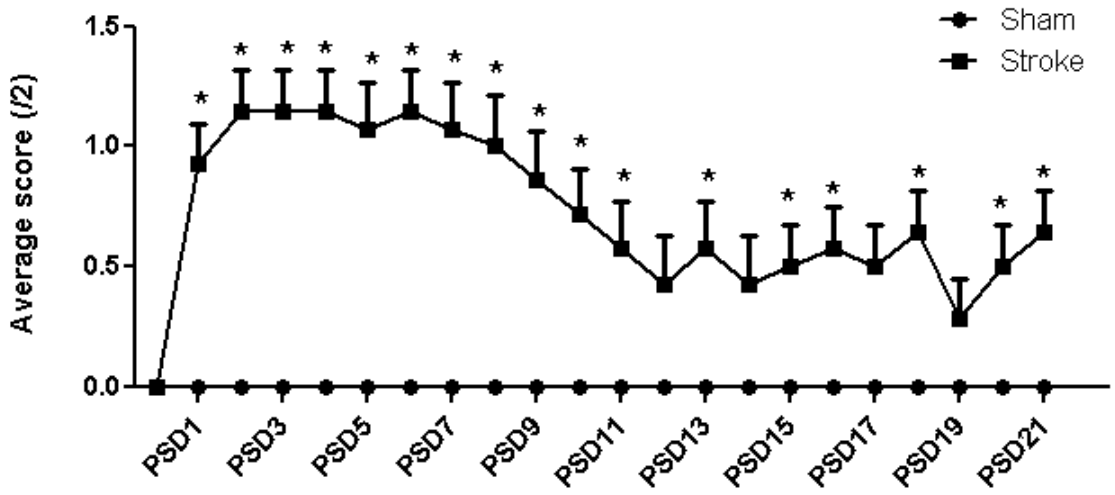


Figure 2.4. Performance on the forelimb postural reflex test. Postural reflex was determined by observing the position of the impaired forelimb while suspending the animal above a flat surface. The reflex was impaired in animals that received ET-1, but not sham surgery. The deficit lasted the duration of the study. n=14/stroke n=6/sham.

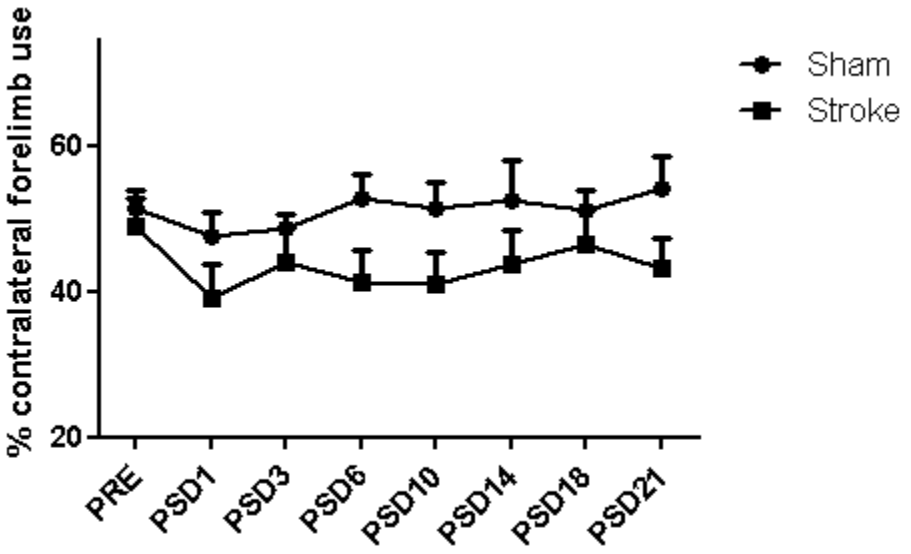


Figure 2.5. Performance on the cylinder test. The Schallert (Schallert et al., 2000) cylinder test of forelimb asymmetry was not significantly impaired following ET-1 surgery compared to Sham surgery. n=14/stroke n=6/sham.

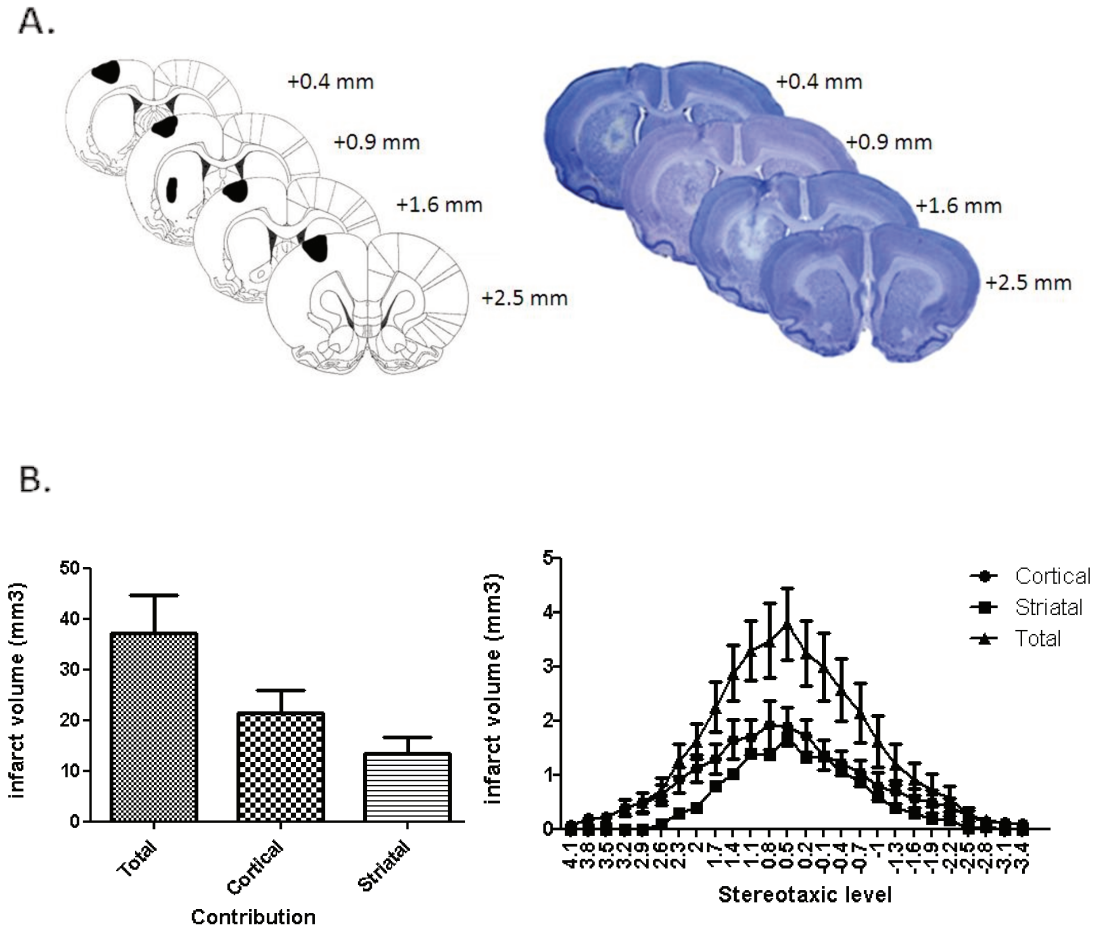


Figure 2.6. Infarct volume and spread. (A) Approximately, the targeted infarct regions based on microinjection locations [in black; adapted from The Rat Brain Atlas (Paxinos and Watson, 2007)], and typical damage produced at these stereotaxic levels (visualized as pale, unstained, or absent areas in the striatum and cortex (B) Volume of the total infarct, as well as the cortical and striatal contribution. (C) Infarct distribution across stereotaxic coordinates. Error bars represent SEM. n=14.

sectioned for complete analysis of the infarct at various stereotaxic levels; a representation of typical damage at each level is presented in Figure 2.6A. The average volume of ischemic damage resulting from this injection protocol was 21.31 ± 4.81 mm^3 (cortical) and 13.36 ± 3.36 mm^3 (striatal), giving a combined total damage volume of 37.2 ± 7.69 mm^3 (Figure 2.6B). The infarct spread and volume at each stereotaxic level is presented in Figure 2.6C.

2.4 DISCUSSION

Mechanistic studies of post-stroke plasticity and rehabilitation require the use of a stroke model that results in consistent, well-defined lesions, with preservation of sufficient neural substrate to support neuroplastic processes. Other groups have evaluated the use of distinct variations of the ET-1 model to generate permanent histopathological damage to forelimb regions of the brain and associated functional impairment (Gilmour et al., 2004; Hewlett and Corbett, 2006; Soleman et al., 2010; Windle et al., 2006). However, no surgical standard currently exists.

The objective of this experiment was to evaluate a modified ET-1 microinjection protocol using tests of forelimb function and histological assessment of the resulting infarct. Stroke often results in a combination of cortical and striatal injury (Cramer, 2003), therefore a combination of cortical and striatal injections were performed. Assessment of the infarct at various stereotaxic levels showed that damage was well-confined to targeted brain regions responsible for forelimb function (Figure 2.6). Infarct variability was lower than that reported using similar ET-1 injection protocols (Hewlett and Corbett, 2006; Hume, 2009; Windle et al., 2006), despite the use of more injections.

This could be due to the use of lower overall injection volumes of a similarly concentrated solution, using more numerous injections [5 injections totalling 4.5 µl of 400 pmol/µl ET-1 in the present study, compared to 3 injections totalling 5 µl (Hewlett and Corbett, 2006) or 6 µl (Windle et al., 2006) of a similarly concentrated solution previously reported].

In addition to measuring infarct volume, characterizing functional deficits that accompany brain injury is important when evaluating a stroke model (Corbett and Nurse, 1998). The results showed that the present injection protocol resulted in forelimb functional deficits that were measurable up to 21 days before recovery occurred (Figure 2.3-2.4). This duration of deficit is similar to previous literature employing these tests (Leasure and Schallert, 2004; Schallert et al., 2000), and longer lasting than previous injection protocols attempted in our laboratory (Hume, 2009).

Interestingly, there was no deficit detected on the cylinder test in this study, similar to previous reports using both rat (Fang et al., 2010) and mouse (Tennant and Jones, 2009) intracerebral ET-1 injection protocols, but contrary to several other studies of both ischemic (Hume, 2009; Schallert et al., 2000; Soleman et al., 2010; Windle et al., 2006) and non-ischemic (Schallert et al., 2000) damage to similar brain regions. The reason for this is unclear; previous cylinder impairments have been reported in the same strain of rat (Hume, 2009; Schallert et al., 2000) thereby ruling out a strain-specific issue. The test is easy to score with a high inter-rater reliability even when administered by inexperienced researchers (Schallert et al., 2000), excluding experimenter error. One possible explanation is the administration of the cylinder test during the animals' less active light cycle. Rats are nocturnal, which could affect motivation to explore in the cylinder during the day (Schallert and Woodlee, 2005). Occasionally, experimenter

interventions were required to stimulate the animals' curiosity to obtain the target 15 rears (e.g. lightly tapping the top of the cylinder, momentarily turning off the lights, etc.). It is possible that these interventions resulted in non-representative bi-tactile rearing responses that may have masked a more robust underlying deficit. However, interventions to motivate performance are suggested by Schallert and Woodlee (2005), and are not believed to affect the limb-use asymmetry score. Nonetheless, for future studies, cylinder testing took place during the dark cycle to eliminate this possibility (see Chapters 3 and 4).

Further issues to note in this study are the ET-1 vehicle used, and the definition of Sham surgery. The current study used sterile water as a vehicle for dissolved ET-1, as has been previously reported (Hewlett and Corbett, 2006; Hicks et al., 2007; Ploughman et al., 2009) including in our laboratory (Hume, 2009). Other appropriate vehicle choices used previously include saline (Hughes et al., 2003; Soleman et al., 2010) and artificial cerebral spinal fluid (Fuxe et al., 1997; Ueki et al., 1993). However, to my knowledge, no systematic comparison of these ET-1 vehicles appears in the literature. In the present study, Sham surgeries did not include craniectomies or injections, similar to others (Kozlowski et al., 1996). Previous literature has shown that even when sham animals undergo skull removal, or skull removal followed by vehicle injection, there is no detectable effect on behaviour (Frost et al., 2006; Gilmour et al., 2004; Hume, 2009; Windle et al., 2006) nor significantly quantifiable lesion volume (Gilmour et al., 2004). Therefore, it is unlikely that the use of other sham surgical procedures would have significantly affected the results of this study.

The ET-1 injection protocol described herein resulted in reproducible lesions to forelimb motor regions, and significant forelimb functional impairments. As such, this

injection protocol was deemed useful for studying post-stroke neuroplasticity and rehabilitation, by preserving more neural tissue to support potential neuroplastic changes than traditional stroke models.

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CHAPTER 3:

EVALUATING A NOVEL MODEL OF VOLUNTARY FORCED USE MOVEMENT THERAPY ON POST-ISCHEMIC FUNCTIONAL RECOVERY AND MARKERS OF NEUROPLASTICITY[2]

"[CIMT] is hard work, but if you put the *work in...there's almost no limit to the amount of recovery that you can have"*

Edward Taub (1931-present)
picture from http://www.newswise.com/articles/remodelling-the-brainrehab-therapy-causes-increase-in-gray-matter

[2] The majority of data presented in this chapter are published in: A novel approach to induction and rehabilitation of deficits in forelimb function in a rat model of ischemic stroke. Acta Pharmacol Sin. 2012, **34**(1): 104-112, with the exception of doublecortin analysis (Results, 3.3.4.3). JLT contributed to experimental design, performed all surgeries, behavioural testing, histology, immunohistochemistry, and prepared the manuscript.

SUMMARY

CIMT is a clinically effective form of rehabilitation. However, modelling CIMT therapy in animals has proven challenging, perhaps due to the use of inappropriate stroke models, animal stress, and lack of behavioural pressure to use the impaired limb. The study performed in Chapter 2 demonstrated that multiple low volume injections of the vasoconstrictor ET-1 to specific stereotaxic coordinates resulted in well-defined and reproducible infarcts and reliable contralesional forelimb impairments. The purpose of the study presented in this Chapter was to test whether a novel appetitively motivated form of forced forelimb use would affect recovery profile, lesion volume, and expression of proteins associated with neuroplasticity in this model of stroke. Following stroke or sham surgery, adult male rats were assigned to receive either daily rehabilitation or a control therapy beginning on PSD 5. Rehabilitation consisted of 30 minutes of voluntary forced use movement sessions using plastic pet activity balls. Rats were tested periodically for 21 days to assess deficit and recovery of forelimb function, and then euthanized for tissue analysis. All animals that received stroke surgery had significant impairments in all functional tests. The rehabilitation therapy resulted in a small but consistent acceleration of forelimb recovery in several functional tests. Stroke increased the proportion of cells expressing the neurotrophin BDNF, as well as the number of doublecortin (Dcx)-expressing cells (indicative of migrating neuroblasts), but these effects were not altered by rehabilitation. There was no change in the expression of the growth inhibiting protein $NOGO_A$. This model of voluntary forced use of the impaired limb warrants further development.

3.1 INTRODUCTION

In addition to being the third leading cause of mortality of North America, the majority of people who survive stroke are left with disabilities (Heart and Stroke Foundation, 2012; Roger et al., 2011). Patients can remain chronically impaired for months to years following a stroke, which vastly impacts quality of life and is associated with a higher rate of post-stroke depression (Burvill et al., 1997; Ramasubbu et al., 1998). While preventive therapies and lifestyle modifications are known to reduce the incidence of the disease (Bousser, 2012), the only effective drug presently available to treat someone experiencing a stroke is tPA. TPA only works in the event of ischemic stroke, by dissolving the offending blood clot to restore blood flow and prevent further ischemic damage. However, it is effective only in a fraction of stroke patients (Zhang and Chopp, 2009) due in part to a narrow therapeutic time window (Madden, 2002; Stemer and Lyden, 2010). Neuroprotective drugs are effective in experimental settings, but to date none have proven clinically effective (O'Collins et al., 2006; STAIR, 1999). As such, many stroke patients continue to rely critically on post-stroke rehabilitation of impairments, and continued efforts toward progression of rehabilitative techniques is warranted to treat the devastating effects of a stroke.

One rehabilitative technique that has been developed to increase use and improve function of upper limbs in survivors of stroke is CIMT. The therapy discourages 'learned non-use' through constraint of the unaffected limb and subsequent forced use of the impaired one for both daily living tasks and intense RTP exercises performed with therapist guidance (Page et al., 2005; Sawaki et al., 2011; Sterr et al., 2002; Taub et al., 2006; Wittenberg et al., 2003; Wolf et al., 2011). A seemingly effective technique

(Nijland et al., 2011), the mechanisms responsible for CIMT-induced recovery of function are not well understood. Valid experimental models are essential for further investigation into this phenomenon.

In recent years, several creative animal models of forced use therapies have been developed (Auriat et al., 2006, 2010; Biernaskie and Corbett, 2001; Biernaskie et al., 2004; DeBow et al., 2003; Fang et al., 2010; Kim et al., 2005; Leasure and Schallert, 2004; Maclellan et al., 2005; Maldonado et al., 2008; Müller et al., 2008; Ploughman et al., 2009; Schabitz et al., 2004; Yang et al., 2003). These have provided important insights into the numerous variables that need to be considered, including the use of paradigms that best model particular and appropriate aspects of CIMT. A major challenge faced by researchers when modelling forced use in animals is that of subject motivation. A main component of CIMT is constraint of the nonparetic limb for most waking hours. Similarly, the intact forelimb can be constrained in rats (DeBow et al., 2003; Leasure and Schallert, 2004; Schabitz et al., 2004), although increased animal stress (Ke et al., 2011; Yanagita et al., 2007) and lack of behavioural pressure to use the impaired forelimb (DeBow et al., 2003) may be problematic. Because of this, some researchers have opted to shift focus away from constraint and toward forced use by other means.

Chapter 2 described the development of an ET-1 stroke method that resulted in reproducible, well-placed lesions, and damage reasonably confined to targeted forelimb motor regions of the brain (see Figure 2.6). Using this procedure, the present experiment evaluated the efficacy of a novel method of appetitively forcing use of the impaired forelimb, intended to circumvent animal stress and behavioural pressure issues. The effects of this therapy on functional recovery, lesion volume, expression of

neuroplasticity-associated proteins (BDNF and $NOGO_A$), and a marker of migrating neuroblasts (Dcx) were investigated.

3.2 METHODS

3.2.1 Experimental animals

Adult male Sprague-Dawley rats (N=46) were purchased from Charles River Laboratories (Montreal, Canada) and single housed on a 12 hr reverse light/dark cycle (lights off at 08:00, on at 20:00). It was found that activity levels affected animals' participation in the rehabilitation, as shown by others (MacLellan et al., 2011), therefore all procedures (surgery, rehabilitation, and behavioural testing) took place during the dark cycle. Animals had ad libitum access to food and water, and weighed between 300-350 g at the time of surgery. All procedures were conducted in accordance with the guidelines of the Canadian Council for Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee.

3.2.2 Surgical procedure

The ET-1 surgical procedure was performed as described in Chapter 2 (see Section 2.2.3). Briefly, rats were placed into an induction chamber filled with isoflurane to induce anaesthesia, which was maintained during surgery at 2%. Once deeply anesthetised, animals were mounted onto a stereotaxic apparatus (David Kopf Instruments, USA), and the scalp was incised and retracted. Small holes were drilled

through the skull at each injection location (see Table 2.1) using a stereotaxically mounted drill (Stoelting Co., USA). Endothelin-1 (Calbiochem, Germany) was injected at each coordinate at a flow rate of 0.5µl/minutes. The scalp was then sutured and the incision site was treated with topical anaesthetic. Body temperature was checked regularly and maintained at 36.0 ± 0.2°C for the duration of surgery using a heating pad. Following surgery, animals were given subcutaneous injections of butorphanol (2.0 mg/kg), returned to their home cage, and allowed to recover. A heating pad was placed under the cage for 1 hour. Sham-operated rats received the same surgical procedure up to but not including the drill holes, and remained anesthetised for the same amount of time as the ET-1 animals (n=11 for each stroke group; n=12 for each sham group).

3.2.3 Rehabilitation

Animals were acclimated to and trained to use commercially available clear plastic pet activity balls (29 cm diameter; Super Pet, USA) (Figure 3.1). Prior to the initiation of the study, pre- and post-stroke ability to move the balls was observed in a separate set of animals, to verify that all ball movement (initiating, propelling, changing direction, and stopping) required the use of both forelimbs. Therefore, animals were forced to evoke use of the impaired forelimb to engage in the post-surgical rehabilitation. Training took place with six exposures over two weeks, and began with exposure to balls that were held stationary on foam docks, then gradually progressed to increasing non-stationary periods of time, and resulted in a final verification of the animal's ability to engage in the rehabilitation.

Figure 3.1. Forced use movement therapy model. Appetitively motivated forced use movement therapy was modelled using clear plastic pet activity balls. Rehabilitation sessions lasted for 30 minutes daily, in a 90 sq ft arena. Control therapy consisted of placing animals into stationary activity balls for 30 minutes daily.

Training was followed by a wash out period of at least 5 days to eliminate any potential neuroprotective effects of this mild exercise (Ploughman et al., 2007). A small number of animals (n=4) were not able to manipulate the balls following the training regimen and were subsequently disqualified from being assigned to a rehabilitation group (randomly assigned instead to either sham or stroke surgery, followed by control therapy).

Following surgery, rats from both surgical groups were randomly assigned to receive voluntary forced use movement therapy (Rehab; n=11 stroke; n=12 sham), or Control therapy (n=11 stroke; n=12 sham) beginning on PSD 5. Rehabilitation sessions lasted for 30 minutes daily, during which the animals were free to move voluntarily around an enclosed 90 sq ft arena (4-6 animals per arena at a time). Sessions were video recorded and later analysed to determine the amount of time each rat spent moving during each 30 minute period. Time spent moving was scored manually by an experimenter blind to the surgical condition and to the test day of each animal. Control therapy consisted of placing rats into stationary balls for 30 minutes each day.

3.2.4 Behavioural testing

For three weeks prior to surgery, animals were handled daily and acclimated to all tests, then pre-surgery scores were recorded. Following surgery, animals were tested regularly as described below until the end of the study at PSD 21. As in Chapter 2, 'deficit' was defined as a mean post-surgical performance significantly worse than the mean sham group performance, and 'recovery' was considered the point at which the mean performance of animals in each experimental group returned to mean sham

performance. In any instance where animals had recovered at a particular time point, then had subsequent testing days with impaired performance, 'recovery' was considered the latest time point at which they consistently performed at sham level. All performance evaluations were conducted by an experimenter blind to the surgical condition and, when possible, to the post surgical testing day.

Several behavioural tests employed in Chapter 2 were used in this study as previously described (see Section 2.2.4; summarized below). Forelimb placing tests (both TFP and VFP) and forelimb postural reflex tests were performed daily under red lights. The cylinder test was performed on PSD 1, 6, 10, 14, 18, and 21. Briefly, for TFP and VFP, the distal portion of the unrestrained forelimb or the corresponding vibrissae was gently brushed against the edge of a table, to measure the ability of the animal to respond by placing its forelimb onto the surface. Functional deficit was determined based on the number of failed forelimb placements on the tabletop out of 5 attempts. Forelimb postural reflex was measured by suspending the animal by the tail and observing the position of the forelimb. A score was assigned from 0 (no deficit) to 2 (abnormal forelimb flexion combined with torso twisting). The cylinder test was performed by placing animals into a clear plastic cylinder and determining the percent usage of the contralateral limb during rearing behaviour.

3.2.4.1 Horizontal ladder test

In the current study, the horizontal ladder test was used to measure forelimb coordination on PSD 1, 6, 10, 14, 18, and 21. Rats were provoked to run across a ladder (1.5 m long) with unevenly spaced rungs (1-3 cm apart) by using a bright light (470 lux)

and white noise (78 dB) at one end of the ladder and an escape box at the other end (Figure 3.2). On each testing day, animals were video recorded during 3 trials, and the average number of foot slips made with the contralesional forelimb was determined by slow motion video analysis.

3.2.5 Histology and infarct quantification

At the end of the study, rats were transcardially perfused with 120 mls phosphate buffered saline (PBS), followed by 120 mls 4% paraformaldehyde in PBS, both at a flow rate of 20 mls/min. Brains were extracted, post-fixed for a further 24 hours in 4% paraformaldehyde, then permeated with 30% sucrose in PBS for 72 hours (or until they were no longer floating in the solution). They were then cryoprotected with Cryomatrix (Thermo Scientific, USA), and stored at -80^{o}C until use. Some brains (n= 4/group) were sectioned (10µm) for immunohistochemical analyses using a Cryostat (Thermo Scientific, USA). Sections were taken from intact tissue caudal and rostral to the damage, as well as anterior, middle, and posterior regions of the infarct itself (in total, at +4.1, +2.3, +0.5, -1.3, -3.1 mm from bregma). Remaining tissue was sectioned at 100 µm and used for infarct quantification using cresyl violet staining. Remaining brains were sectioned at 100 µm for cresyl violet staining only. Tissue was mounted on slides, dried overnight, and stored at -20^{o}C until use.

Infarct quantification was performed as described previously (see Section 2.2.5). Photographs of every third section (i.e. 300 µm apart) were assessed for infarct damage using Image J software (NIH, USA) by tracing the area of injury as defined by lack of cresyl violet staining (Luke et al., 2004). The total volume of brain injury was

Figure 3.2. Horizontal ladder test. Animals were provoked to run across a horizontal ladder of unevenly spaced rungs by placing aversive stimuli at one end and an escape box at the other. Slow motion video analysis was used to determine the number of foot slips made with the impaired forelimb.

determined by summing the area of damaged tissue recorded from each section and multiplying that value by the distance between the measured sections.

3.2.6 Immunohistochemistry

Unless otherwise stated, 10 μm sections from the 5 brain regions indicated above were used for immunohistochemical analyses. Before each protocol, tissue was subjected to antigen retrieval by heating at 90°C for 20 minutes in sodium citrate buffer (pH 6.0). Negative controls were prepared by omitting the primary antibodies.

Slides were rinsed in PBS, incubated with 3% H_2O_2 for 20 minutes, followed by blocking solution (5% goat serum in PBS) for 2 hours at room temperature. They were then incubated with rabbit anti-BDNF (1:500; #AB1779, Millipore, USA) for 48 hours or rabbit anti-$NOGO_A$ (1:1000; #sc-25660, Santa Cruz, USA) for 24 hours at 4°C. Following incubation with primary antibody, sections were washed in PBS (3 x 15 minutes), and incubated with secondary antibody (1:200; biotinylated goat-anti-rabbit IgG; Vector, USA) for 1 hour at room temperature. Slides were then incubated with avidin biotin complex (ABC; Vector Laboratories, USA) for 30 minutes and visualized using a solution of 3, 3'-diaminobenzidine tetrahydrochloride and H_2O_2 (DAB; Vector Laboratories, USA). Following immunohistochemical staining, slides were counterstained with hematoxylin to visualize the cell nuclei.

Sections (10 μm) from +2.3, +0.5, and -1.3 mm relative to bregma only were processed for the presence of Dcx+ cells, indicative of newly generated neurons. This subset of sections was chosen from the larger sample of 5 sections, because they would include infarcted tissue based on infarct spread observed in Chapter 2 (see Figure 2.6).

Sections were incubated in 3% H_2O_2 for 20 minutes, then in blocking solution (10% horse serum in 0.2% Triton X-100 in PBS) for 1 hour at room temperature, followed by goat anti-Dcx (1:250; #c-18, Santa Cruz Biotechnologies, USA) at 4°C for 24 hours. The following day, they were incubated in the presence of secondary antibody (1:200; biotinylated horse anti-goat IgG, Vector, USA) for 1 hour, then ABC for 30 minutes. Following washing, bound antibody was visualized with a solution of DAB and H_2O_2; sections were then counterstained with hematoxylin to visualize cell nuclei.

3.2.7 Cell counting

BDNF and $NOGO_A$ expression in various regions of interest (ROI) in the ipsilesional and contralesional hemisphere (and corresponding regions of sham sections) was quantified by calculating the percentage of cells in each region expressing the protein of interest [e.g. (# BDNF positive cells/total # cells) X 100]. Counting was performed at 400 x magnification (each ROI measuring 0.35 x 0.25 mm) in three regions along the visible lesion boundary, one in the ipsilesional striatum, and 4 equivalent regions of the contralesional hemisphere (see Figure 3.3A).

The number of Dcx-positive cells was determined by counting ROIs on the lesion border, ipsilesional striatum and SVZ, and corresponding contralesional cortex, striatum and SVZ (see Figure 3.3B). Photographs were taken at 200 x magnification and ROIs measured 0.67 x 0.54 mm. All counting was performed by an observer blind to treatment group.

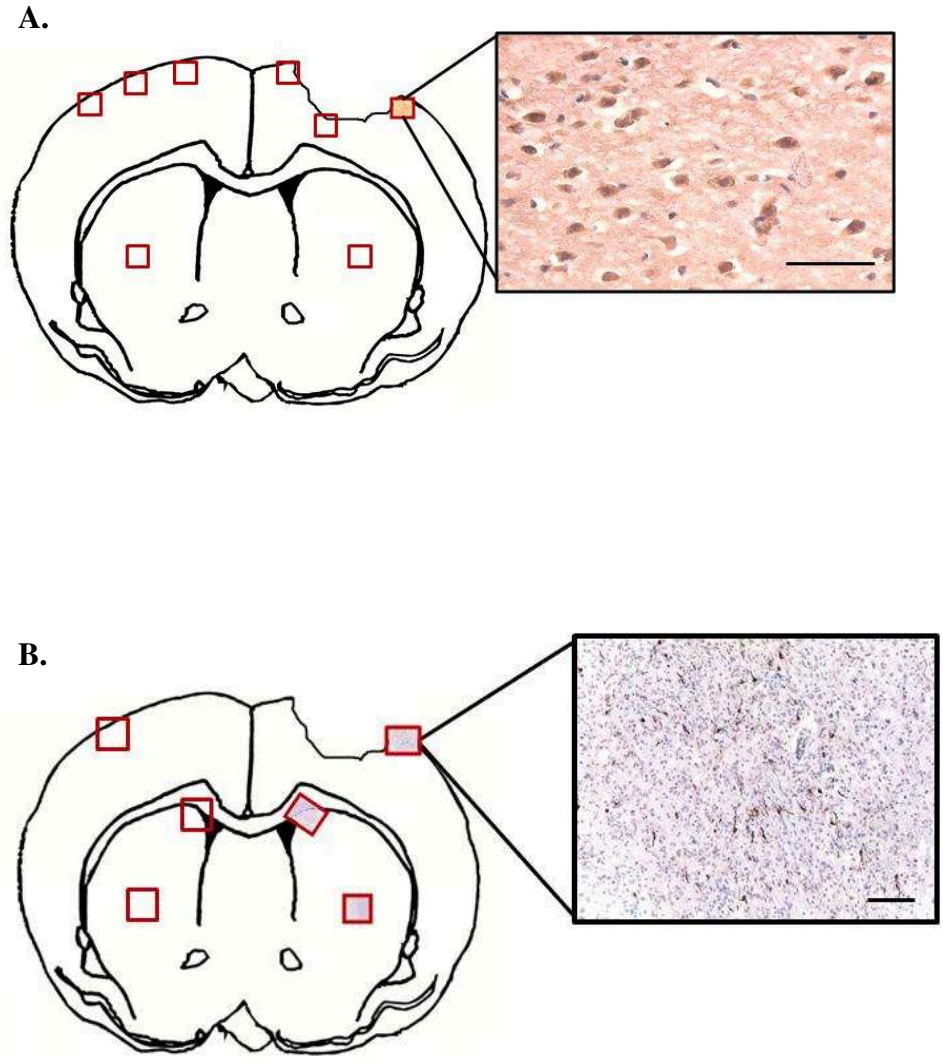


Figure 3.3. Immunohistochemistry regions of interest. (A) BDNF and $NOGO_A$ expression were examined from 8 ROIs (0.35 x 0.25 mm) as indicated by the red boxes. (B) Doublecortin expression was examined from 6 ROIs (0.70 x 0.56 mm). Scale bars = 100 µm.

3.2.8 Statistical analyses

Statistical analyses were performed using PAWS Statistics (v. 18; SPSS inc., Chicago, USA). Behavioural data were analysed using RM 2-way ANOVA. Where the assumptions of sphericity were violated (Mauchly's test; $p<0.05$) the Greenhouse–Geisser correction was applied (degrees of freedom are reported to the nearest integer; see Section 2.2.6). Subsequent Bonferroni post-hoc tests were used to determine differences between groups on specific days. Immunohistochemistry was analysed using 2 way ANOVAs (for surgical condition and rehabilitation group). Infarct volumes were compared using independent t-tests. Values are expressed as mean ± SEM.

3.3 RESULTS

3.3.1 Rehabilitation

While Rehab was an independent variable in the present study, it was of interest to compare the overall activity levels of Stroke/Rehab and Sham/Rehab animals. All animals spent an equal amount of time moving during the 30 minute rehabilitation sessions on any given day ($p > 0.05$), however the amount of time declined steadily over the course of the study from an average of 18 minutes on PSD 5 to an average of 12 minutes by PSD 21 in both groups (20% decrease; $p <0.0001$) (Figure 3.4).

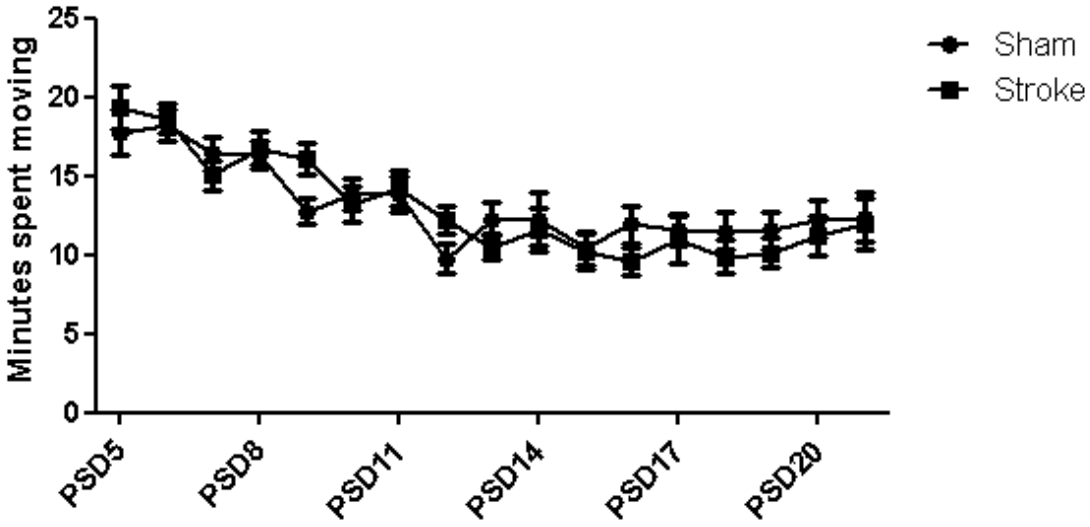


Figure 3.4. Voluntary rehabilitation intensity. Intensity of voluntary rehabilitation was analysed as time spent moving for each daily rehabilitation session. There were no significant differences between Sham and Stroke animals on any day. Rehabilitation intensity declined over the course of the study. n= 11-12/ group

3.3.2 Behavioural tests

3.3.2.1 Forelimb placing tests

Prior to surgery, all animals had equivalent performance on both the TFP and VFP tests, scoring 5/5 without error. Following surgery, there was no ipsilesional impairment in any group. On the contralesional tactile-stimulated forelimb placing test, there was a significant effect of group ($F_{3,42}$=43.8; p<0.0005), time ($F_{8,335}$=23.0; p<0.0005), and interaction of group x time ($F_{24,335}$=7.46; p<0.0005). Post hoc analysis revealed that both ischemia groups had a significant deficit following stroke surgery. The Stroke/Rehab group recovered at PSD 16 (p=0.944) while control animals recovered at PSD 21 (p=1.00) (Figure 3.5A).

On the vibrissae-stimulated placing test, there was a significant effect of group ($F_{3,42}$ =32.4; p<0.0005), time ($F_{7,287}$ =18.1; p<0.0005), and interaction of group x time ($F_{20,287}$ =5.63; p<0.0005). Both stroke groups were significantly impaired following surgery. Rehabilitation animals remained impaired for the duration of the study (p=0.022), while control animals recovered at PSD 20 (p=0.122) (Figure 3.5B).

3.3.2.2 Forelimb postural reflex test

Prior to surgery, all animals had a normal score of '0' on the forelimb postural reflex test. Following surgery, animals from both ischemia groups showed a significant deficit (p<0.001). There was a significant effect of group ($F_{3,42}$ =28.7; p<0.0005), time ($F_{10,80}$=9.58; p<0.0005), and interaction of group x time ($F_{29,411}$ =4.12; p<0.0005).

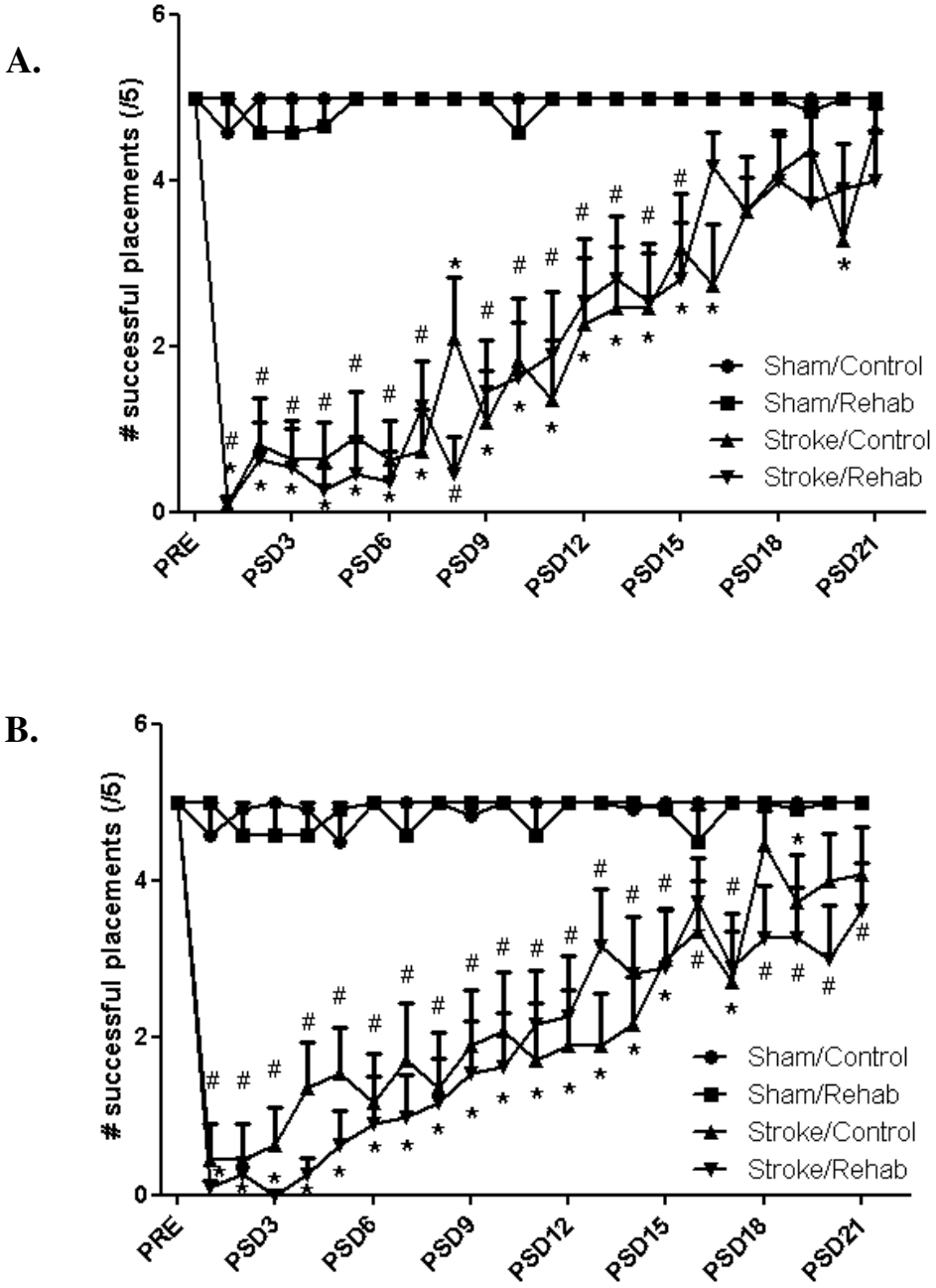


Figure 3.5. Performance on the forelimb placing tests. (A) Tactile-stimulated forelimb placing was impaired in both stroke groups following endothelin-1 administration. Animals undergoing forced use movement therapy recovered function 5 days sooner than control therapy animals. (B) Similarly, vibrissae-stimulated forelimb placing was impaired in both groups following endothelin-1 injection. Control animals had spontaneously recovered by PSD 20, while Rehab animals remained impaired until PSD 21. *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$. n=11/stroke groups, n=12/sham groups.

Animals in the Stroke/Rehab group were impaired until PSD 19 ($p=0.240$) while those from the Stroke/Control group were impaired until PSD 20 ($p=0.098$) (Figure 3.6).

3.3.2.3 Cylinder Test

Before surgery, all animals used the contralesional forelimb for approximately 50% of exploratory movement while rearing in the cylinder. Following surgery, there was a significant effect of group ($F_{3,42}$ =13.2; $p<0.0005$), time ($F_{4,185}=4.56$; $p<0.0005$), and interaction of group x time ($F_{13,185}$ =4.97; $p<0.0005$). Stroke animals showed a significant decrease in the use of the contralateral limb on the first post-surgical day ($p<0.01$) that recovered by PSD 14 ($p=0.100$) in the Rehab group, while the deficit in control therapy animals recovered by PSD 21 ($p=0.076$; Figure 3.7).

3.3.2.4 Horizontal ladder test

Prior to surgery, all animals were able to cross the ladder with a minimal average number of foot slips (0.4±0.4). Following surgery, there was a significant effect of group ($F_{3,42}=3.01$; $p=0.041$), time ($F_{4,190}=7.01$; $p<0.0005$), and interaction of group x time ($F_{4,190}=3.33$; $p<0.0005$). The average number of foot slips with the contralesional forelimb was significantly greater in both of the ischemia groups ($p<0.01$) (1.9±0.45 for Stroke/Control; 1.6±0.34 for Stroke/Rehab) relative to shams (0.33±0.13 for Sham/Control; 0.5±0.17 for Sham/Rehab). This impairment recovered by PSD 6 in Rehab animals ($p=0.273$), and recovered by PSD 10 in Control animals ($p=0.057$) (Figure 3.8).

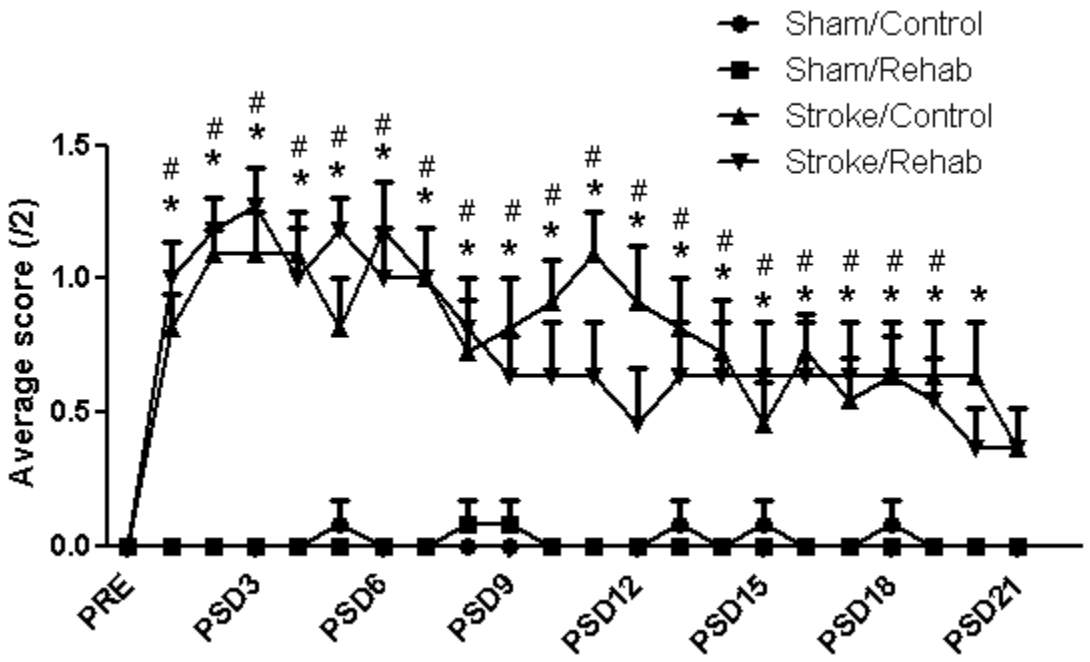


Figure 3.6. Performance on the forelimb postural reflex test. The position of the impaired forelimb as the animal was suspended above the home cage was scored from 0 (no deficit) to 2 (severe deficit). Following surgery, animals in the stroke group had a significant impairment. Animals in the Rehab group recovered by PSD 20; control animals recovered by PSD 21. *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$. n=11/stroke groups, n=12/sham groups.

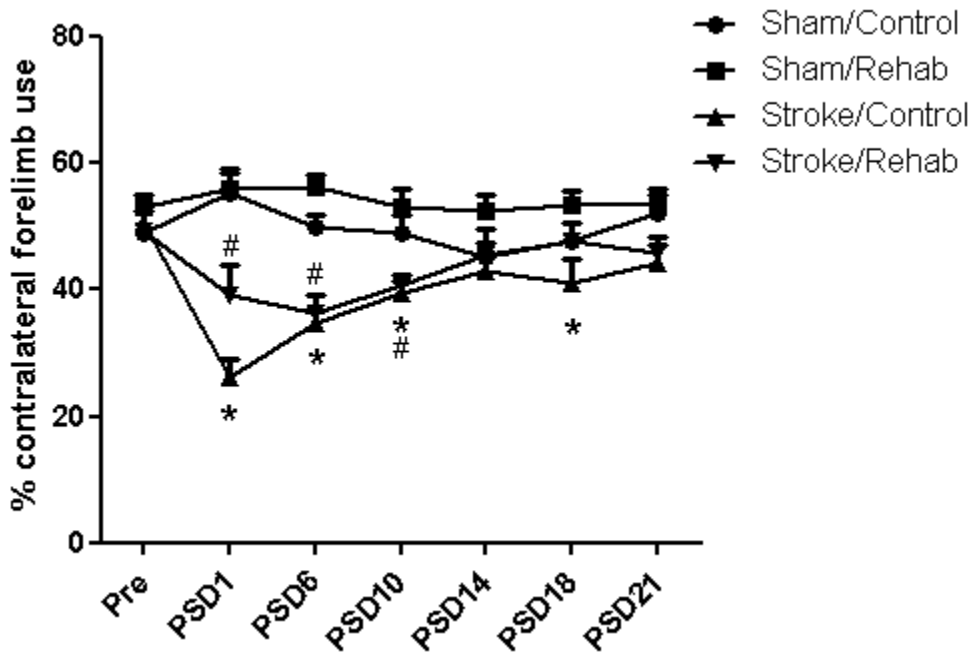


Figure 3.7. Performance on the cylinder test. Following surgery, animals in both stroke groups used the impaired forelimb significantly less than sham controls. Rehab animals recovered by PSD 14; control animals recovered by PSD 21. *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$. n=11/stroke groups, n=12/sham groups.

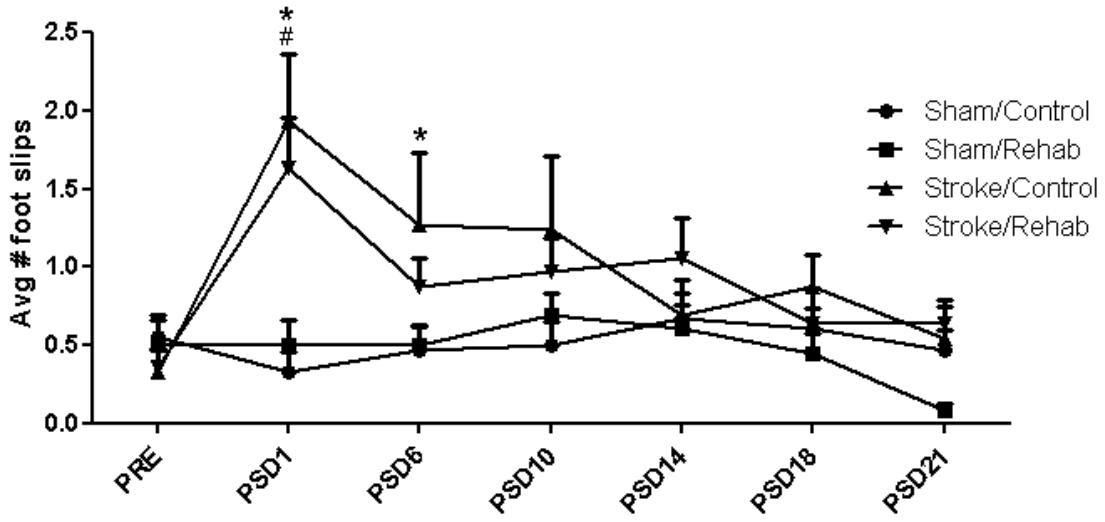


Figure 3.8. Performance on the horizontal ladder test. Prior to surgery, all animals were able to cross the horizontal ladder with few foot slips. Following surgery, there was a significant deficit in both stroke groups. By PSD 6, the Rehab animals had recovered, while controls remained impaired until PSD 10. *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$. n=11/stroke groups, n=12/sham groups.

3.3.3 Infarct quantification

Cresyl violet histology for infarct quantification revealed that there was no significant difference in the total infarct volume between groups. Animals receiving control therapy had similarly sized infarcts compared to animals receiving rehabilitation (12.2 ± 3.0 mm3 and 10.7 ± 2.0 mm3, respectively) (p=0.412) (Figure 3.9A). The infarct spread and volume at each stereotaxic level is presented in Figure 3.9B.

3.3.4 Immunohistochemistry

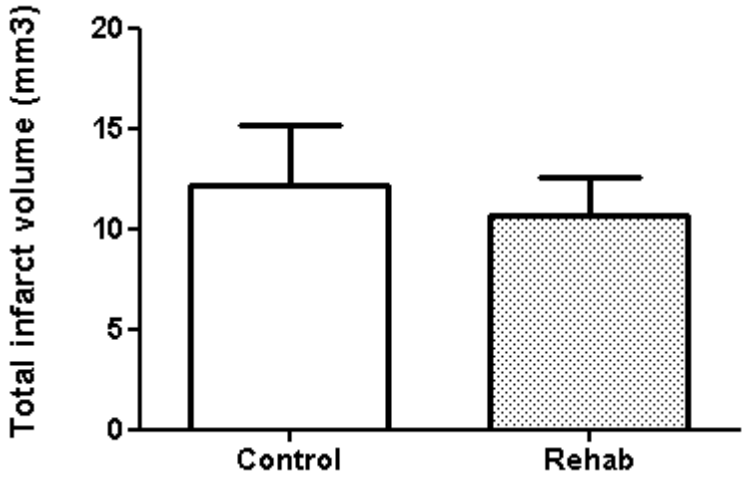
3.3.4.1 BDNF

The proportion of cells expressing BDNF was determined from a total of 8 areas of interest encompassing both the ipsilesional and contralesional hemispheres (see Section 3.2.7). In the ipsilesional hemisphere of both stroke groups, there was an increase in the percent cells expressing BDNF ($p<0.05$), however this ratio remained unaffected by rehabilitative therapy (Figure 3.10B). In the contralesional hemisphere there were no significant differences in the percentage of cells expressing BDNF (data not shown).

3.3.4.2 $NOGO_A$

The proportion of cells expressing $NOGO_A$ was determined from the same regions of interest as BDNF. There were no significant differences between the stroke

A.



B.

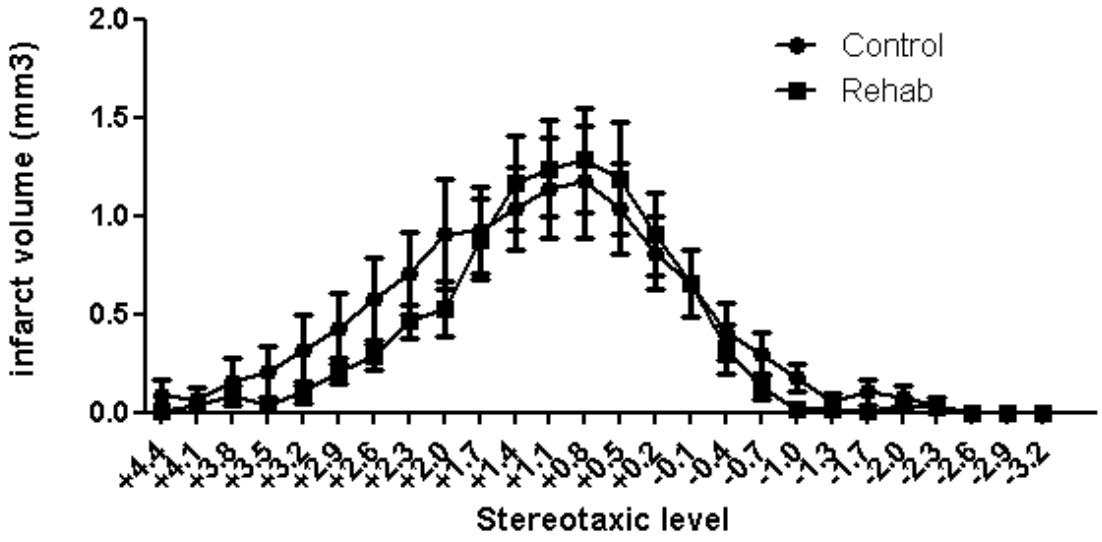
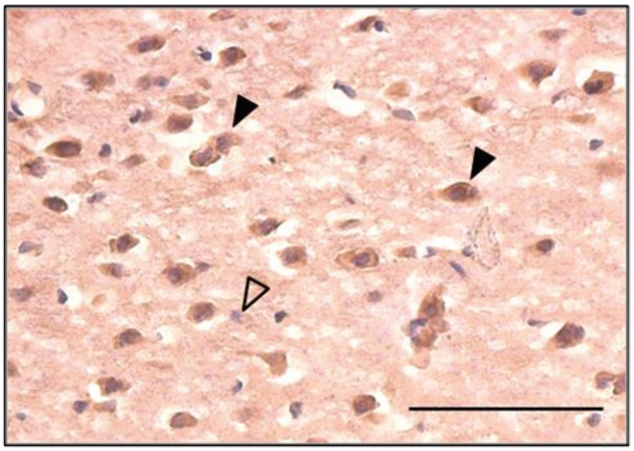


Figure 3.9. Infarct volume and spread. (A) Volume of the total infarct was the same in the animals that received Rehab and control therapy. (B) The distribution of the infarct across stereotaxic levels was similar between groups. n=11/group.

A.



B.

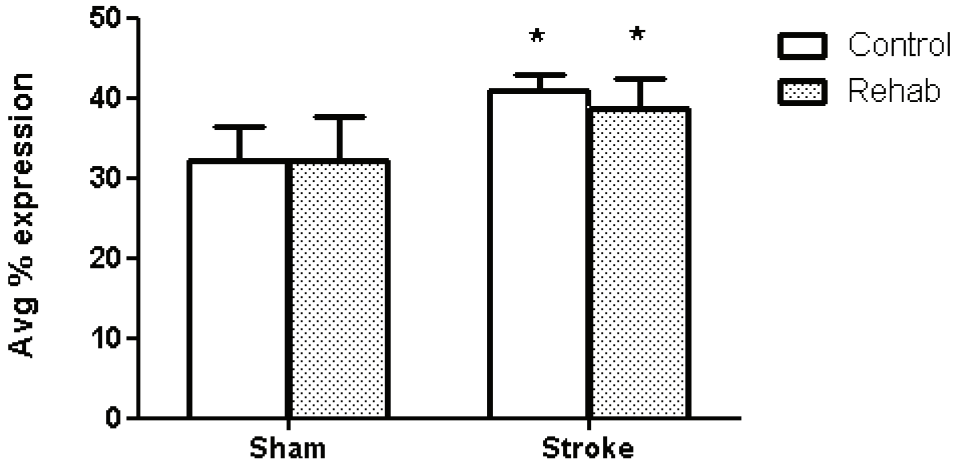


Figure 3.10. Proportion of ipsilesional cells expressing BDNF. (A) Expression of the neurotrophin BDNF was examined using immunohistochemistry. Cells expressing BDNF were stained brown (solid arrowheads), while nuclei of all cells were visualized with hematoxylin (empty arrowhead). Scale bar = 100 µm. (B) Stroke surgery caused a significant increase in the proportion of cells that expressed BDNF. This proportion was not affected by rehabilitation. n=4/group *p<0.05.

and sham groups in either the contralesional (data not shown) or ipsilesional hemispheres (Figure 3.11B).

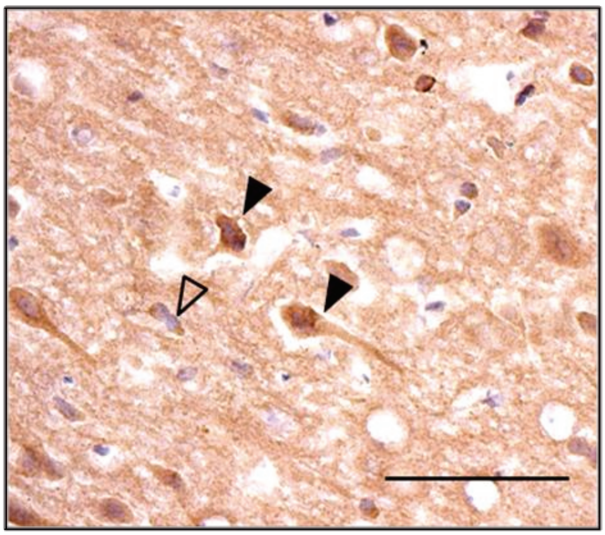
3.3.4.3 Neuroblasts

The total number of Dcx+ cells was summed in each of the ipsi- and contralesional SVZ, cortex, and striatum. Animals in the both stroke groups exhibited significantly more ipsilesional Dcx+ cells than sham animals ($p<0.02$), however rehabilitation did not affect Dcx expression (Figure 3.12B).

3.4 DISCUSSION

While prophylactic lifestyle modifications and neuroprotection represent preventive measures for the damage resulting from stroke, current therapeutic options are severely limited, and many people continue to live with varying degrees of disability. As such, it is important to better understand how rehabilitation can influence neuroplasticity, and ultimately, improve function. Arm impairment following stroke represents one of the most common disabilities in stroke survivors (Jorgensen et al., 1995a, 1995b; Kelly-Hayes et al., 1998). Forcing use of the impaired arm using CIMT can result in improved functional recovery in stroke patients. To better understand the mechanisms underlying CIMT-assisted recovery, a number of experimental studies have investigated forced use using animal models. Because of the intense but generally voluntary nature of clinical rehabilitation, animal models of rehabilitation are

A.



B.

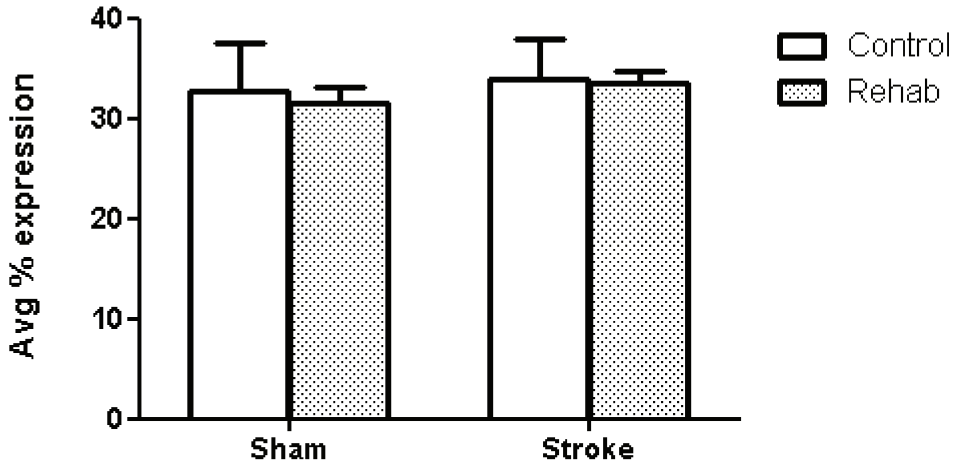


Figure 3.11. Proportion of ipsilesional cells expressing $NOGO_A$. (A) Expression of $NOGO_A$, a growth inhibiting protein, was examined using immunohistochemistry. Cells expressing $NOGO_A$ were stained brown (solid arrowheads), while nuclei of all cells were visualized with hematoxylin (empty arrowhead). Scale bar = 100 µm. (B) Neither stroke surgery nor rehabilitation affected the proportion of cells that expressed $NOGO_A$. n=4/group.

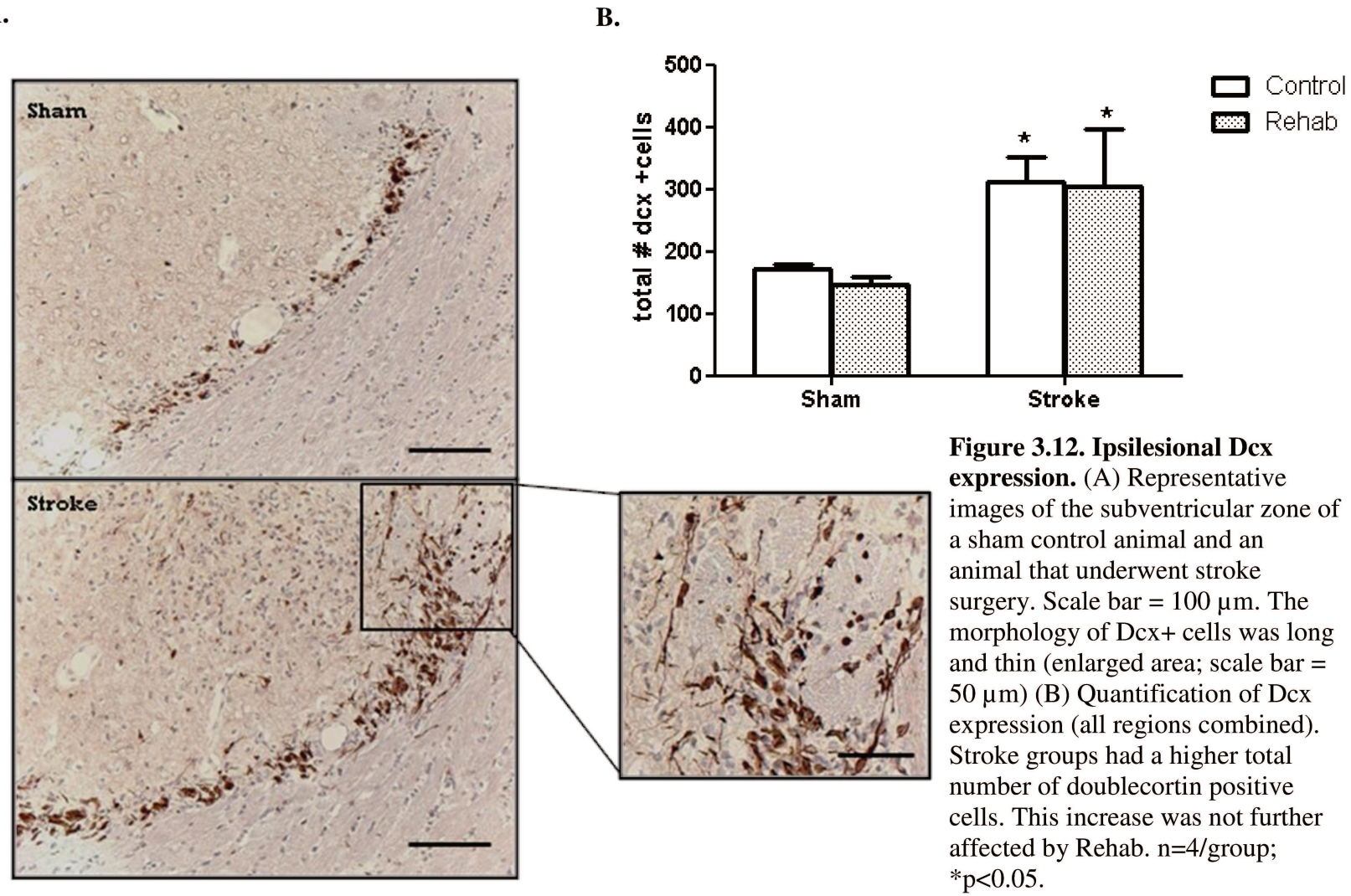


Figure 3.12. Ipsilesional Dcx expression. (A) Representative images of the subventricular zone of a sham control animal and an animal that underwent stroke surgery. Scale bar = 100 µm. The morphology of Dcx+ cells was long and thin (enlarged area; scale bar = 50 µm) (B) Quantification of Dcx expression (all regions combined). Stroke groups had a higher total number of doublecortin positive cells. This increase was not further affected by Rehab. n=4/group; *p<0.05.

challenging, and necessitate focus on particular aspects of clinical therapy (see Section 1.7.2). Post-ischemic exposure of rats to various task-specific (Biernaskie and Corbett, 2001; DeBow et al., 2003; Maldonado et al., 2008) or nonspecific (Johansson and Ohlsson, 1996; Ke et al., 2011; Marin et al., 2003; Ohlsson and Johansson, 1995) forelimb activity has been shown to improve function. However, forced use of the affected forelimb by plaster casting of the unaffected limb after photothrombotic ischemia did not accelerate motor recovery (Schabitz et al., 2004), Similarly, constraint of the intact forelimb as a singular approach following hemorrhagic stroke did not accelerate recovery, while the addition of exercises that further forced use of the unconstrained (impaired) forelimb resulted in improvement (DeBow et al., 2003). Notably, some experimental models of forced limb use in rodents have reported increased damage and worsened functional recovery as a result of rehabilitation (Bland et al., 2000; DeBow et al., 2004; Humm et al., 1998; Kozlowski et al., 1996). The reasons for these discrepancies are unclear, but could be due to (1) the use of stroke models that produce inconsistent infarcts (Carmichael, 2005), (2) the intensity of forced use rehabilitation employed (MacLellan et al., 2011), (3) varying behavioural pressures (DeBow et al., 2003), and in some cases, (4) animal stress (Kirkland et al., 2008).

The present study evaluated the effects of a novel voluntary forced use therapy model on upper extremity function, lesion volume, and expression of proteins involved in neuroplasticity. This is the first time pet activity balls have been used in the context of post-stroke forelimb rehabilitation, although a similar protocol was employed by Moroz et al. (2004) to model mild exercise following 6-hydroxydopamine lesion.

Several of the tests used in the present study were also used in the experiment described in Chapter 2. The ET-1 surgery resulted in a similar deficit on the vibrissae

and tactile stimulated forelimb placing tests, significant for 19 and 20 days in Chapter 2 (see Figure 2.3) and 20 and 21 days in the present study (see Figure 3.5). The forelimb postural reflex test also resulted in a significant impairment, this time lasting up to 20 days (see Figure 3.6) similar to the 21 days in Chapter 2 (see Figure 2.4). In the current study there was a significant deficit in performance on the cylinder test in both stroke groups. This test had not detected a deficit in Chapter 2 (see Figure 2.5). A possible explanation for this discrepancy is that in the present study, all testing was performed in the more active dark cycle, which may have increased the proportion of naturally elicited rears (see Section 2.4). An additional test added to the battery in this study was the horizontal ladder test, which revealed a significant stroke-induced deficit lasting up to PSD 10.

Voluntary forced use rehabilitation caused a modest but significant acceleration in functional recovery using several assessments of forelimb function (Figures 3.3-3.6), without exacerbating damage (Figure 3.9). Interestingly, while rats spent most of the rehabilitation session moving earlier in the study, their engagement in activity ball usage declined throughout the duration of the study (Figure 3.2). This is surprising, as rats given free access to running wheels for several hours per day naturally increase their activity over time (Leasure and Grider, 2010; Makatsori et al., 2003; Persson et al., 2004; Risedal et al., 2002; Shyu et al., 1984). Rehabilitation is believed to be most effective when it is of increasing intensity, both in clinical (Carr and Shepherd, 2011) and experimental (Ke et al., 2011) studies, thus declining participation in this experiment is a concern. It is possible that animals became increasingly accustomed to and disinterested in the rehabilitation over time. Increasing voluntary participation in the therapy may result in more marked improvement in recovery. During rehabilitation, 4-6

animals were placed in the arena at a time. An alternative approach would be to give each animal a single arena to move around, with other animals nearby, which may increase curiosity to explore and counteract the decline in voluntary engagement over time.

As highlighted in Section 3.2.3, navigation of the ball around the arena requires the use of all four limbs. Interestingly, the amount of time spent rolling the exercise ball did not differ between ischemic and control rats (Figure 3.4). Thus, despite marked deficits on contralesional forelimb function on multiple tests (Figure 3.5-3.8) impaired rats continually used the limb during rehabilitation. A similar outcome was noted by Marin et al. (2003), who found that post-stroke voluntary wheel running was similar between animals with large infarcts and those with little or no damage. A possible explanation for the discrepancy in use of the impaired limb for activity ball movement versus behavioural testing may involve the engagement of central pattern generators (CPGs). CPGs involve neural networks in the spinal cord that can produce rhythmic movements such as walking, even when isolated from the brain (Grillner and Wallen, 1985). The nervous system is capable of generating CPGs following complete deafferentation and paralysis (Mackay-Lyons, 2002). Thus, ball rolling movement may comprise both voluntary and automatic components, involving a combination of cerebral and spinal inputs.

Stroke caused a significant increase in the proportion of ipsilesional cells expressing BDNF, similar to other reports (Béjot et al., 2011a, 2011b; Madinier et al., 2009), but this proportion was not affected by rehabilitation (Figure 3.8). Other rehabilitative paradigms have been shown to increase the production of BDNF (Kim et al., 2005; MacLellan et al., 2011; Ploughman et al., 2009) using quantitative analyses

such as gene expression (Ploughman et al., 2009) or quantitative protein expression (Kim et al., 2005; MacLellan et al., 2011). While the proportion of BDNF-expressing cells in the present study was unaffected by rehabilitation, each cell may have increased absolute BDNF production. Analysis using protein quantification methods (e.g. Western blot) is required to investigate this possibility.

Doublecortin is a protein required for neuronal migration of newly generated neurons, and is commonly examined when investigating neurogenesis (Komitova et al., 2005; Plane et al., 2008; Wang et al., 2007). In this study, stroke resulted in an increase in the number of Dcx+ cells in the ipsilesional SVZ, cortex, and striatum compared to sham surgery. It has been shown by several groups that brain injury such as ischemia results in reactive neurogenesis (Carmichael, 2008; Gu et al., 2000; Jiang et al., 2001; Jin et al., 2001; Kernie and Parent, 2010; Leasure and Grider, 2010; Parent et al., 2002; Wang et al., 2007; Xiong et al., 2010; Zhang et al., 2001), and functional recovery can be attenuated if neurogenesis is impeded (Jin et al., 2010; Raber et al., 2004; Sun et al., 2012). In the current study, rehabilitation did not further affect the number of Dcx+ cells (Figure 3.12B), while other rehabilitation paradigms have been shown to increase neurogenesis (Briones et al., 2005; Nilsson et al., 1999; Wurm et al., 2007). However, direct comparisons cannot be made between those results and the ones presented herein, due to the use of different techniques. Notably, studies examining neurogenesis usually use injection of bromodeoxyuridine (BrdU), a thymidine analogue that becomes incorporated into dividing cells, as a marker of neurogenesis. In the present study, however, the decision to look for evidence of neurogenesis was made after the tissue had been collected, thereby rendering BrdU injections impossible. Instead, evidence of immature neurons was investigated using Dcx labelling. This method is not directly

comparable to studies that incorporate BrdU for several reasons. Firstly, this method only provides a snapshot of the Dcx+ cells that were present at the time of sacrifice, while BrdU injection protocols usually examine neurogenesis over a window of time. Further, BrdU labels all phenotypes of newborn cells, including astrocytes. Therefore, an increase in the number of BrdU+ cells does not necessarily reflect the number of newly formed neurons. A study by Leasure and Grider (2010) highlighted the importance of considering the phenotype of newly born cells, by showing that nearly all of the BrdU+ cells in the peri-infarct region were either undifferentiated or non-neuronal in nature (Leasure and Grider, 2010). Lastly, while Dcx labelling is commonly used in combination with BrdU to confirm the identity and specificity of newly born cells (Breunig et al., 2007), it is not a sufficiently robust marker to prove neurogenesis, considering evidence that it can be expressed by mature neurons undergoing structural plasticity (Nacher et al., 2001). Nonetheless, the Dcx+ cells observed in the present study had characteristic morphology of migratory neuroblasts (long and thin, with few processes; see Figure 3.12), and were located almost exclusively between the SVZ and the areas of damage. Further confirmatory studies specifically aimed at investigating the role of neurogenesis following stroke, as well as long-term cell survival and integration, would be of interest.

$NOGO_A$ is a protein that inhibits axonal outgrowth following CNS injury (Cheatwood et al., 2008), and treatment with anti-$NOGO_A$ enhances post-ischemic neuroplasticity and functional recovery (Papadopoulos et al., 2002). A detailed profile of $NOGO_A$ expression following MCAo revealed dynamic temporal and spatial changes in expression of the protein following ischemia (Cheatwood et al., 2008). Rehabilitation combined with a $NOGO_A$ antagonist, NEP 1-40, promotes functional recovery (Fang et

al., 2010), indicating that it may play an important role in post-ischemic neuroplastic events. In the present study, it was hypothesized that accelerated recovery of function following rehabilitation would be associated with a decrease in the expression of this growth-inhibiting protein. However, the proportion of cells expressing $NOGO_A$ in the chosen regions of interest was not affected in either hemisphere by either stroke or rehabilitation at PSD 21. The intricate temporal profile of $NOGO_A$ expression has not yet been characterized in the ET-1 model, so it is possible that examination at a different time point, or different region of the brain, may have revealed alterations. Furthermore, as highlighted in the discussion of BDNF expression (see above) it is impossible to surmise based on the technique used in this study whether there was a quantitative decrease in the amount of protein being expressed from the (unchanged) proportion of cells.

In summary, the use of a novel approach to voluntary forced use therapy results in a modest acceleration in functional recovery on some tests, through mechanisms unrelated to increasing the proportion of BDNF-expressing cells or the number of new neurons. Further refinement of this method could prove useful in developing strategies to study and improve post-ischemic rehabilitation and neuroplasticity.

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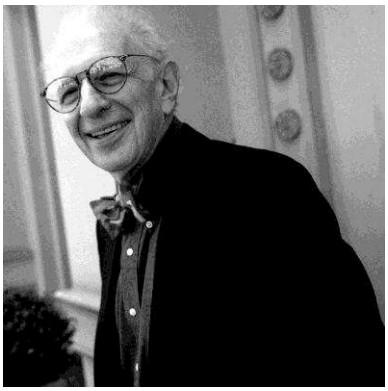
CHAPTER 4:

EVALUATING A REFINED MODEL OF VOLUNTARY FORCED USE REHABILITATION ON POST-ISCHEMIC FUNCTIONAL RECOVERY AND MARKERS OF NEUROPLASTICITY



"[CIMT] is a terrific rehabilitation therapy....and it speaks to the fact that the plastic capability of the brain...is even greater *than we thought"*

Eric Kandel (1929-present)

picture from http://www.rmanyc.org/events/load/1548

SUMMARY

Constraint induced movement therapy, which forces use of the impaired arm following unilateral stroke, improves functional recovery. Issues with animal motivation make modelling CIMT challenging. Chapter 3 described how a novel rehabilitative paradigm that appetitively engaged the impaired forelimb following ischemic injury led to a modest acceleration in recovery and did not affect lesion volume. The purpose of this study was to investigate the effects of a modified voluntary regimen on recovery profile, with the addition of a task specific reaching exercise. Adult male rats were subjected to stroke or sham surgery. Stroke animals were then assigned to either daily rehabilitation or control therapy beginning on PSD 3. Rehabilitation consisted of 30 minutes of voluntary activity ball sessions (as evaluated in Chapter 3) followed by 30 minutes of voluntary task-specific movement using reaching boxes. Animals were tested weekly to assess deficit and recovery of forelimb function for 28 days, including evaluation in a test of skilled reaching. At the end of the experiment, animals were euthanized and tissue was examined for infarct volume, BDNF expression, and Dcx+ cells. The modified voluntary rehabilitation resulted in acceleration of forelimb recovery using several tests, and a significant increase in the number of Dcx+ cells. There was no difference in the proportion of BDNF expressing cells, or the number of microglia at PSD 28. However, there was a shift in the cellular origin of the BDNF being expressed, resulting in significantly more non-neuronal/non-astrocytic BDNF, presumed to be of microglial origin.

4.1 INTRODUCTION

Almost 850,000 North Americans experience a stroke each year (Heart and Stroke Foundation, 2012; Roger et al., 2011). Because of logistical constraints, many stroke patients do not receive the clot-dissolving drug tPA, leading to a high rate of impairment (Madden, 2002; Stemer and Lyden, 2010; Zhang and Chopp, 2009). As a result, it has been suggested that stroke has a greater disability impact than any other chronic disease (Adamson et al., 2004). Patients can remain chronically impaired for months to years following stroke, which vastly impacts quality of life for both survivors and caretakers (Burvill et al., 1997; Ramasubbu et al., 1998). As such, many stroke patients continue to rely critically on post-stroke rehabilitation, and continued efforts toward progression of rehabilitative techniques is warranted to improve treatment of the devastating effects of a stroke.

Rehabilitation has traditionally focused on preventing functional impairment from worsening, and learning to compensate for loss of function (Sandin, 2012). However, with an evolving understanding of the brain's post-injury potential to reorganize, emphasis has shifted toward the goal of neuroreparation. One rehabilitative approach that has been developed to improve function of the upper limb in survivors of stroke is CIMT. The therapy discourages 'learned non-use', a phenomenon whereby movement is initially suppressed due to failure and adverse consequences encountered when a subject attempts to use the affected limb (Taub et al., 2006). This results in persistent compensatory behaviours and subsequent suppression of use of the impaired limb, even when function may eventually be possible (see Figure 1.7). Through constraint of the unaffected limb and subsequent forced use of the affected one, CIMT

encourages positive feedback about the limb's functional potential. Generally, the constraint device is worn for most waking hours during a two week period, and is accompanied by intensive RTP performed daily using the (unconstrained) impaired limb (Sawaki et al., 2011; Wittenberg et al., 2003). RTP and shaping exercises are designed to engage participants in meaningful functional activities, with measurable progressions for which they receive positive feedback.

Chapter 3 described how a novel animal model of appetitive forced use of the impaired forelimb using commercial pet activity balls resulted in a modest acceleration of functional recovery. The purpose of this study was to further develop the model by the addition of an appetitively-motivated, task-specific component (similar to the RTP portion of CIMT). This study evaluated whether this modified model could result in accelerated functional recovery, as well as impact infarct volume, expression of BDNF, number of microglia, and number of Dcx+ cells after 28 days.

4.2 METHODS

4.2.1 Experimental animals

Adult male Sprague-Dawley rats (N=32) were purchased from Charles River Laboratories (Montreal, Canada) and single housed on a 12 hr reverse light/dark cycle (lights off at 08:00, on at 20:00). As in Chapter 3, all procedures took place during the dark cycle, as activity levels affected animals' participation in the rehabilitation. Unless otherwise stated, animals had ad libitum access to food and water, and weighed between 300-350 g at the time of surgery. All procedures were conducted in accordance with the

guidelines of the Canadian Council for Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee.

4.2.2 Surgical procedure

The ET-1 surgical procedure was performed as described in the previous Chapters (for full description, see Section 2.2.3). Briefly, rats were placed into an induction chamber pre-filled with isoflurane to induce anaesthesia, which was later maintained during surgery using 2% isoflurane in oxygen. Once deeply anesthetised, animals were mounted onto a stereotaxic apparatus (David Kopf Instruments, USA), and the scalp was incised and retracted with clamps. Small holes were drilled through the skull at each injection location (see Table 2.1) using a stereotaxically mounted drill (Stoelting Co., USA). In this study, lesions were made contralateral to the preferred paw, as determined by staircase test training (described below, in Section 4.2.4.1). Endothelin-1 (Calbiochem, Germany) was injected at each location at a flow rate of 0.5µl/min. After the injections, the scalp was sutured and the incision was treated with topical anaesthetic. Body temperature was maintained at 36.0 ± 0.2°C for the duration of surgery using a heating pad. Following surgery, animals were given subcutaneous injections of butorphanol (2.0 mg/kg), returned to their home cage, and allowed to recover. A heating pad was placed under the cage for 1 hour. Sham-operated rats received the same surgical procedure up to but not including the drill holes, and remained anesthetised for the same amount of time as the ET-1 animals (n=5 sham; n=18 stroke).

4.2.3 Rehabilitation

Following surgery, ischemia animals began either a modified rehabilitation therapy (Rehab; n=9) or Control therapy (n=9) on PSD 3. Daily rehabilitation sessions consisted of 30 minutes of activity ball movement, followed by 30 minutes of task specific movement. A similar rehabilitation program used in Chapter 3 had no effect on the performance or histology of sham animals (see Section 3.3), therefore to reduce the number of animals required for the present study, sham surgical controls remained untreated. Animals were trained to use the activity balls as described in Chapter 3 (see Section 3.2.3) and to perform the task specific reaching exercise described below. Those who would not engage in either portion of the rehabilitation following training were disqualified from the rehabilitation groups (n=6).

4.2.3.1 Activity ball rehabilitation

Prior to surgery, animals were trained as described in Chapter 3 to use commercially available clear plastic pet activity balls (29 cm diameter; Super Pet, USA) (Figure 4.1A). During the 30 minute daily rehabilitation sessions, animals were allowed to move around an enclosed 40 sq ft arena (1 animal per arena). Sessions were video recorded and a subset of time points (PSD 3, 14, 28) was analysed using ANY-maze© software (Stoelting Co., USA) to estimate rehabilitation intensity (as distance moved) and to assess group variability. Control therapy animals were placed in stationary balls for 30 minutes each day.

A. **B.**

Figure 4.1. Modified forced use movement therapy model. Rehabilitation involved two components: (A) General movement therapy was modelled using clear plastic pet activity balls. Rehabilitation sessions lasted for 30 minutes daily, in a 40 sq ft arena. (B) Reaching boxes measured 7 cm wide x 3 cm deep x 7 cm tall, with the platform holding pellets at a height of 3 cm. Boxes were placed into the home cage, and filled on either the left or right side, such that the animal was prompted to reach voluntarily with his contralesional forepaw for up to 30 minutes/day. Control animals were placed into stationary balls for 30 minutes/day and given the equivalent number of pellets without having to reach.

4.2.3.2 Task specific rehabilitation

Immediately following daily activity ball sessions, Stroke/Rehab animals were given a further 30 minutes of task-specific reaching rehabilitation. Reaching boxes filled with banana-flavoured sucrose pellets were secured in the animals' home cages. Prior to the initiation of the study, animals were acclimated to the reaching boxes with three exposures, and then the ability to use the boxes was verified. Reaching boxes were constructed from Plexiglas and measured 7 x 3 x 7 cm, with a central 1 cm wide gap to reach through and a 3 cm high platform for pellets (Figure 4.1B). For each animal, the ipsilesional side of the box (opposite the impaired forelimb) was filled with 100 pellets such that reaching was encouraged with the impaired side. Stroke/Control animals were provided with the average number of pellets consumed by the Stroke/Rehab animals each day, without requiring reaching.

4.2.4 Behavioural testing

For three weeks prior to surgery, animals were handled daily and acclimated to all tests, then pre-surgery scores were recorded. Following surgery, animals were tested weekly until PSD 28. As in the previous studies, 'deficit' was defined as a mean post-surgical performance significantly worse than the sham group and 'recovery' was considered the point at which the mean performance of animals in each experimental group returned to mean sham group performance. In any instance where animals had recovered at a particular time point, then had subsequent testing days with impaired performance, 'recovery' was considered the latest time point at which they consistently

performed at sham level. All performance evaluations were conducted by an experimenter blind to the surgical condition and, when possible, to post-surgical testing day.

Forelimb placing tests were performed as described in Chapter 2 (see Section 2.2.4.1); the horizontal ladder test was performed as described in Chapter 3 (see Section 3.3.2.4). Briefly, for TFP and VFP, the distal portion of the unrestrained forelimb or the corresponding vibrissae was gently brushed against the edge of a table, to measure the ability of the animal to respond by placing its forelimb onto the surface. Performance was determined based on the number of correct forelimb placements on the table top out of 5 attempts. For the horizontal ladder test, animals ran across a 1.5 m long ladder comprised of unevenly spaced rungs (1-3 cm apart) for 3 trials. The average number of foot slips made with the contralesional forelimb was determined by slow motion video analysis.

4.2.4.1 Montoya staircase test

The Montoya staircase test (Montoya et al., 1991) was used to assess deficit and recovery of skilled reaching in this study. Rats were first trained for 14 consecutive days prior to the experiment. During the training period, the test was administered as described below while animals were restricted to 4 hours/day ad libitum feeding (all animals maintained ≥90% body mass). Any animals that were unable to perform the staircase test following the training period were excluded from the study (n=8). Following the 14 day training period, rats were returned to regular ad libitum feeding. Training results were used to determine paw preference for each animal, as defined by

the paw with which the animal consistently reached more pellets on the last 3 days of training.

To perform the test, animals were placed into a rodent motility staircase apparatus that consisted of a box and plinth (Campden Instruments, UK) (Figure 4.2). On either side of the plinth were 7 steps of increasing reaching difficulty, each baited with 3 banana-flavoured sucrose pellets, for a total of 21 pellets accessible to each forelimb. The animals were placed into the staircase apparatus for 15 minutes, after which the number of pellets remaining on each step was recorded, as well as the maximum step that had been reached (as evidenced by disturbed pellets).

4.2.5 Histology and infarct quantification

At PSD 30 rats were deeply anaesthetized with 4% isoflurane in oxygen. Following rapid decapitation, brains were extracted and post-fixed for 72 hours in 10% buffered neutral formalin + 30% (w/v) sucrose. Tissue was cryoprotected using Cryomatrix (Thermo Scientific, USA) and stored at -80^{o}C until use. Tissue was sectioned using a Cryostat (Thermo Scientific, USA) at 50 µm throughout most of the brain; several sets of 10 µm sections were taken from the damaged brain region (+0.8 mm from bregma) for immunohistochemical analysis.

Sections (50 µm) from all animals in the two stroke groups were then stained with cresyl violet, placed on a standard lightbox, and photographed. Pictures of every sixth section (i.e. 300 µm apart) were assessed for infarct damage using Image J software (NIH, USA). Ischemic injury was defined and estimated as described in the previous Chapters (see Sections 2.2.5, 3.2.5).

Figure 4.2. Staircase Test. Animals were tested for skilled reaching by being placed into the staircase apparatus for 15 minutes each testing day. The number of pellets consumed and the maximum step of 7 increasingly difficult levels was recorded. Steps were baited with banana flavoured sucrose pellets.

4.2.6 Immunohistochemistry

Sections (10 µm) were processed for markers of neuroplasticity (n=4/group, unless otherwise stated). After rinsing in PBS, antigen retrieval was performed by heating the slides at 90^{o}C for 20 minutes in sodium citrate buffer (pH 6.0). Slides were then cooled and rinsed in PBS, then used for immunofluoresence or immunochemistry as described below. Negative controls were prepared by omitting the primary antibodies.

4.2.6.1 Immunofluoresence

Immunofluoresence was performed to determine the co-localization of BDNF with cell phenotype markers. Sections were incubated in blocking solution (10% goat serum/1% BSA/0.1% Triton X-100 in PBS) for 2 hours, followed by a combination of primary antibodies overnight [1:250 mouse anti-NeuN, #AB377; 1:400 chicken anti-GFAP, #AB5541; 1:100 rabbit anti-BDNF, #AB1779, Millipore, USA] at 4°C. Sections were washed 3 x 10 minutes in PBS+0.1% Tween. They were then incubated with the following Alexa Fluor conjugated secondary antibodies: Alexa 350 goat anti-mouse IgG (1:100), Alexa 594 goat anti-chicken IgG (1:200), and Alexa 488 goat anti-rabbit IgG (1:200; all from Invitrogen, USA) for 1 hour. Fluorescence signals were detected with a Zeiss AxioObserver.Z1 inverted fluorescent microscope with an Apotome system, scanning microfine slices (1.9 µm) at excitation/emission wavelengths of 323/340, 585/615, and 470/505 nm for NeuN, GFAP, and BDNF labelling, respectively.

4.2.6.2 Diaminobenzidine Tetrachloride

Sections were incubated in 3% H_2O_2 for 20 minutes, then in blocking solution (10% goat or horse serum/1% BSA/0.1% Triton X-100 in PBS) for 1 h at room temperature, followed by rabbit anti-BDNF (1:500, #AB1779; Millipore, USA), rabbit anti-Iba1 (1:500, #019-1974; Wako Chemicals, USA), or goat anti-Dcx (1:250, #c-18; Santa Cruz Biotechnologies, USA) antibodies at 4°C overnight. The following day, they were incubated in the presence of secondary antibody (1:200 biotinylated goat anti-rabbit or horse anti-goat) for 1 hour, then ABC for 2 hours (all from Vector Laboratories, USA). Following washing, sections were visualized with a solution of DAB and H_2O_2 (Vector Laboratories, USA), and counterstained with hematoxylin to visualize cell nuclei (Dcx and BDNF only). Verification of microglial reaction in the infarct using anti-Iba1 was performed using n=4 for each stroke group with a single sham subject for comparison.

4.2.7 Cell counting

The proportion of cells expressing BDNF [(# BDNF+ cells/total # cells) X 100] and the number of Iba1+ cells was determined in the ipsilesional and contralesional hemisphere (and corresponding regions of sham sections) using DAB-labelled sections. Counting was performed at 400 x magnification (each ROI measuring 0.35 x 0.25 mm) along the cortical lesion boundary, in the ipsilesional striatum, and in 2 equivalent regions of the contralesional hemisphere (Figure 4.3). The number of Dcx+ cells was determined by counting cells in ROIs on the lesion border, ipsilesional striatum and

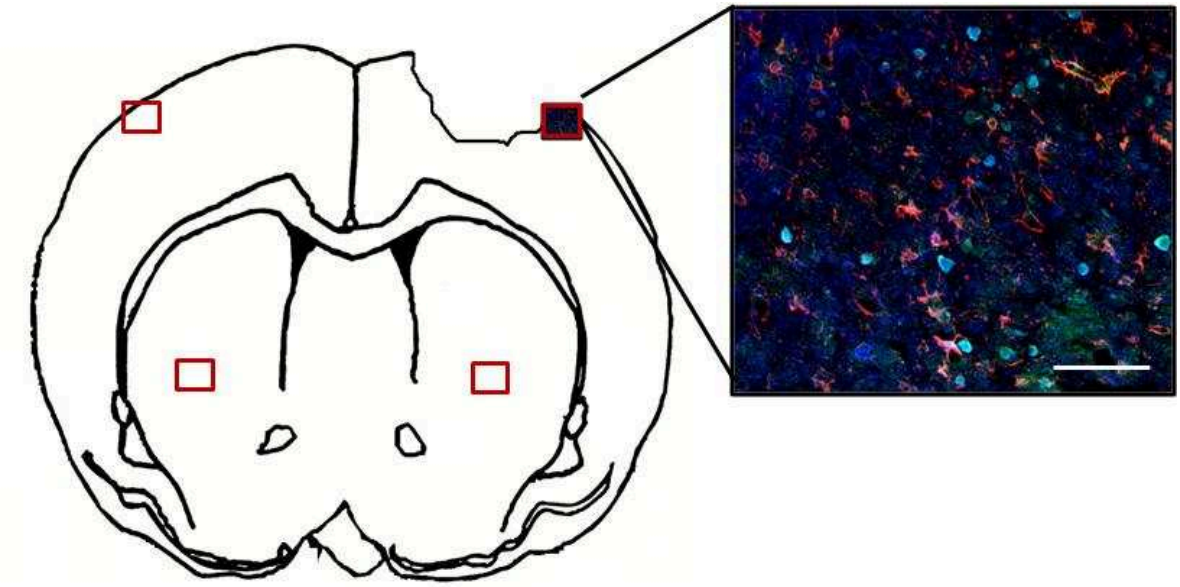


Figure 4.3. Immunohistochemistry regions of interest. The proportion of Iba1+ cells and BDNF expressing cells, as well as triple labelling to determine the co-localization of BDNF with NeuN and GFAP, was determined from two ipsi- and two contralesional ROIs (0.70 x 0.56 mm). Scale bar= 100 µm.

SVZ, and corresponding contralesional cortex, striatum and SVZ. Counting was performed at 200 x magnification and ROIs measured 0.67 x 0.54 mm (see Figure 3.3B).

For immunofluoresence, the number of immunopositive cell bodies was counted at the same four regions, at 200 x magnification (ROIs measured 0.70 x 0.56 mm). Counting was performed by an observer blinded to treatment group, using Zeiss Axiovision 4.7 image analysis software. A co-labeled BDNF+/phenotypic marker+ cell was defined as expressing both immunolabels within the same optical section.

4.2.8 Statistical analyses

Statistical analyses were performed using PAWS Statistics (v. 18; SPSS inc., Chicago, USA). Behavioural data were analysed using RM ANOVA. Where the assumptions of sphericity were violated (Mauchly's test; $p<0.05$) the Greenhouse–Geisser correction was applied (see Section 2.2.6; degrees of freedom are reported to the nearest integer). When warranted, subsequent Bonferroni post-tests were used to determine differences between groups on specific days. Rehabilitation intensity and immunohistochemistry (with the exception of Iba1 analysis) were analysed using one way ANOVAs, with subsequent Bonferroni post-hoc tests to determine group differences. Infarct volumes and Iba1 expression were compared between groups using independent t-tests. Values are expressed as mean ± SEM.

4.3 RESULTS

4.3.1 Rehabilitation

The intensity of voluntary engagement in the activity ball paradigm was analysed from video recordings using a subset of early, middle, and late time points (PSD 3, 14, and 28). Mean performance (distance moved) of rats was not significantly different between days, averaging 160 meters/session (Figure 4.4A). The same subset of time points was used to assess participation in task-specific reaching. Animals consumed significantly fewer pellets on the first day of rehabilitation ($p=0.001$), but consumption was equal on days 14 and 28 ($p=0.332$) (Figure 4.4B).

4.3.2 Behavioural tests

4.3.2.1 Forelimb placing tests

Following surgery, all ischemic animals had a significant contralesional functional deficit compared to controls when responding to tactile stimulation. Analysis revealed a significant effect of group ($F_{2,20}=12.2$; $p<0.0005$), time ($F_{3,48}=15.8$; $p<0.0005$), and an interaction of group x time ($F_{5,48}=3.52$; $p=0.009$) beginning on PSD 1 ($p<0.001$; Figure 4.5A). Rats that were receiving rehabilitation had recovered by PSD 14 ($p=0.169$), compared to PSD 21 for control animals ($p=0.112$).

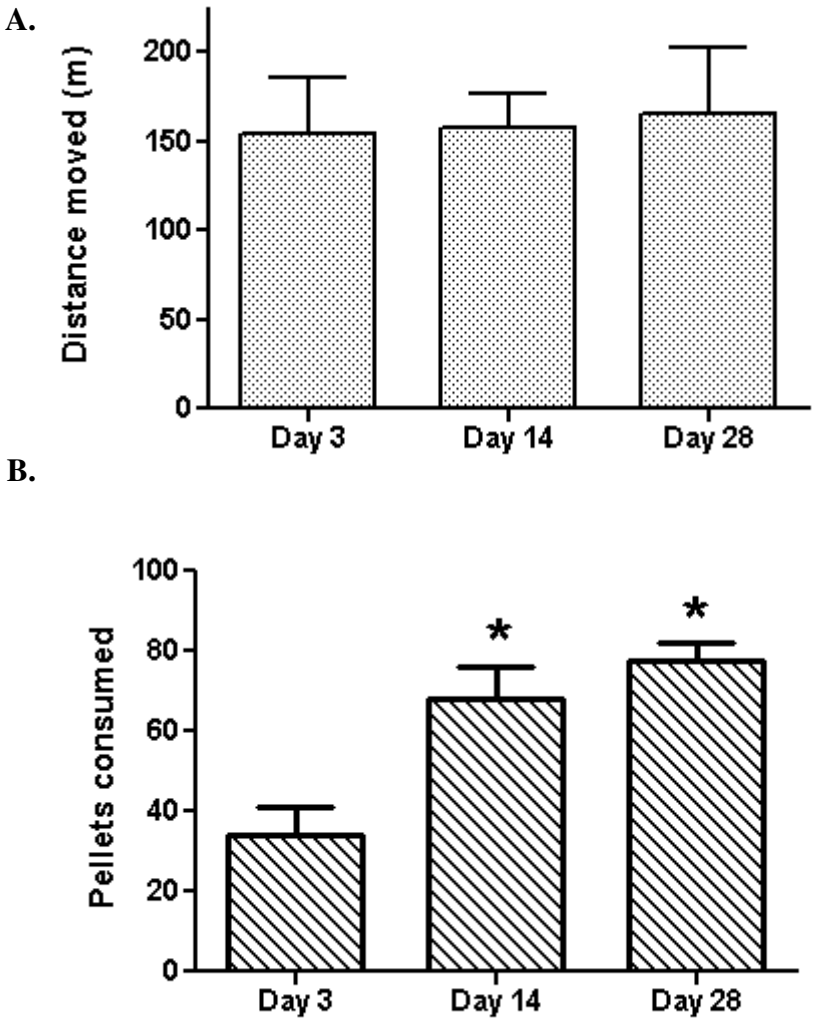


Figure 4.4. Rehabilitation intensity. A subset of time points were sampled to determined the intensity of voluntary rehabilitation engagement. (A) The average distance moved in the activity balls during 30 minute voluntary rehabilitation sessions was the same at all time points sampled. (B) On the first day of rehabilitation, animals reached fewer pellets in the RTP task, however this stabilized by the second post surgical week. n=9; *p<0.05.

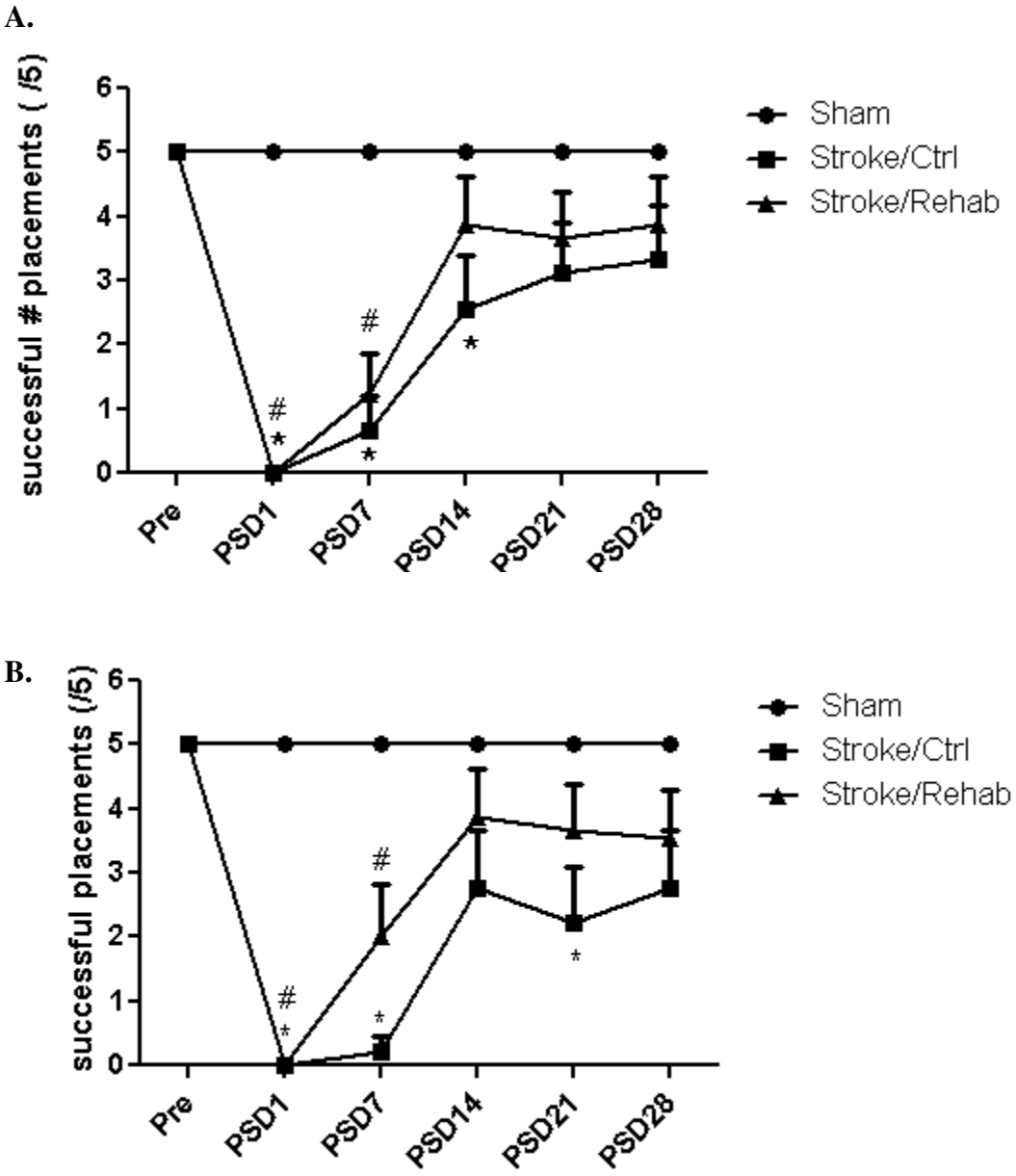


Figure 4.5. Performance on forelimb placing tests. (A) Tactile-stimulated forelimb placing of animals in both stroke groups was significantly impaired compared to shams; this performance recovered earlier in animals receiving rehabilitation. (B) Similarly, vibrissae stimulated placing of animals in both stroke groups revealed a significant impairment which recovered earlier in the rehabilitation group. n=5/shams; 9/stroke groups; *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$.

Similarly, all animals that received stroke surgery had a significant functional deficit in vibrissae-stimulated forelimb placing following surgery. There was a significant effect of group ($F_{2,20}$=12.9; p<0.0005), time ($F_{3,65}$=13.9; p<0.0005), and an interaction of group x time ($F_{6,65}$=3.43; p=0.004). Rats receiving rehabilitation had sustained deficits until PSD 14 (p=0.370) compared to control animals that had deficits until PSD 28 (p=0.084; Figure 4.5B).

4.3.2.2 Horizontal ladder test

Prior to surgery, all animals were able to cross the ladder with a small average number of foot slips (1.3 ± 0.11). On PSD 1, the number of foot slips with the contralesional forelimb was significantly greater in both ischemia groups (p<0.001). There was a significant effect of group ($F_{2,20}$=4.27; p=0.029), time ($F_{5,100}$=9.38; p<0.0005), and interaction of group x time ($F_{10,100}$=2.39; p=0.014). Further analysis revealed that by PSD 7, both groups had recovered performance to Sham level (Figure 4.6).

4.3.2.3 Montoya staircase test

On PSD 1, both ischemia groups had a significant deficit in reaching performance with respect to maximum step reached (p<0.001). There was a significant effect of group ($F_{2,20}$=10.3; p=0.001), time ($F_{5,100}$=6.19; p<0.0005), and interaction of group x time ($F_{10,100}$=2.52; p=0.010). Animals that received rehabilitation recovered to sham performance by PSD 14, whereas control treatment animals had not recovered by

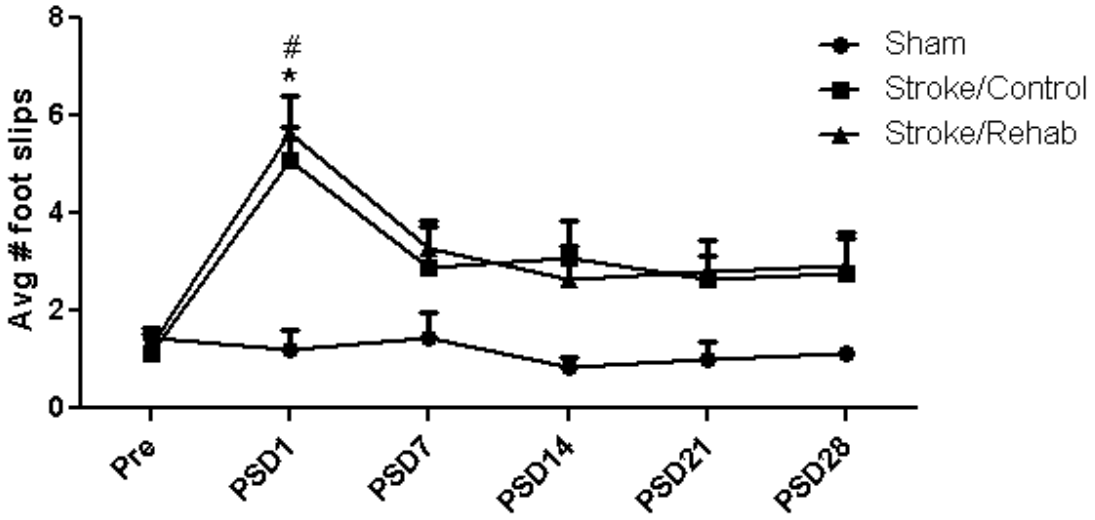


Figure 4.6. Performance on the horizontal ladder test. Animals in both stroke groups had a significant impairment in running performance, as defined by the number of foot slips while crossing the ladder on the first post-surgical day. This impairment recovered in both groups by the following testing day. n=5/shams; 9/stroke groups; *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$.

the end of the study (PSD 28 p=0.018; Figure 4.7A). Results are presented as percentage of pre-surgery performance. Following surgery, both ischemic groups also had a significant deficit with respect to the number of pellets consumed ($p<0.001$). Again, there was a significant effect of group ($F_{2,20}=15.2$; $p<0.0005$), time ($F_{3,60}=12.1$; $p<0.0005$), and a group x time interaction ($F_{6,60}=5.01$; $p<0.0005$). However, further analysis revealed that neither stroke group recovered by the end of the study ($p<0.0005$; Figure 4.7B).

4.3.3 Histology and infarct quantification

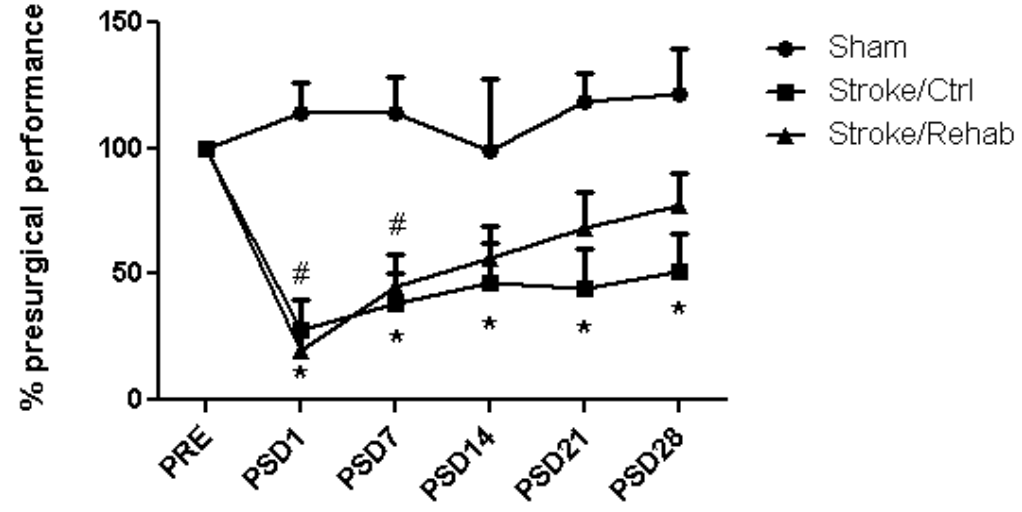
Infarct volume was not significantly different between the two stroke groups ($p=0.23$), and measured 10.6 ± 5.7 mm^3 (SEM) for animals receiving control therapy and 25.7 ± 8.6 mm^3 for those receiving rehabilitation (Figure 4.8).

4.3.4 Immunohistochemistry

4.3.4.1 BDNF

The proportion of cells expressing BDNF was determined in both the ipsilesional and contralesional hemispheres using immunohistochemistry. There were no significant differences between any of the groups in either hemisphere ($p>0.05$) (data not shown). The same regions were then examined for the cellular origin of BDNF by visualizing co-localization of BDNF with NeuN (mature neurons) or GFAP (astrocytes), or by

A.



B.

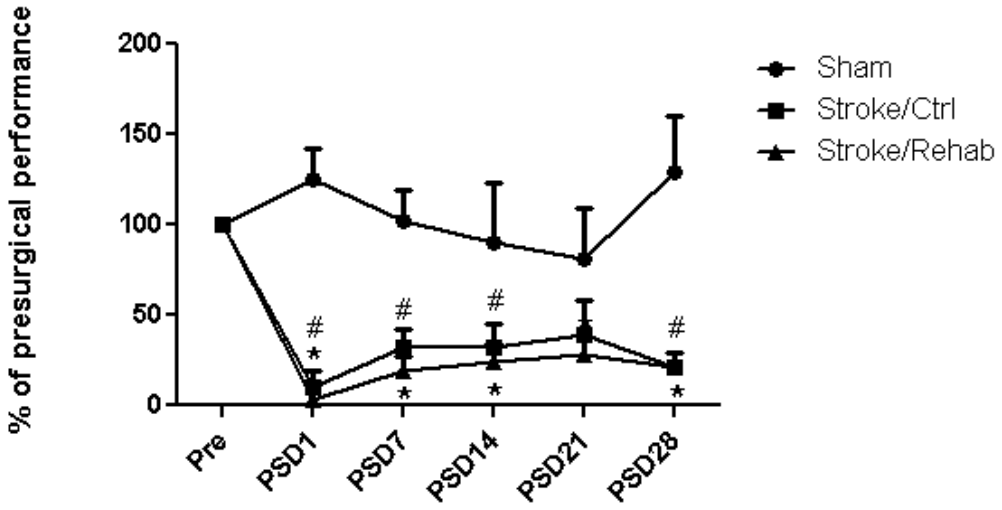


Figure 4.7. Performance on the staircase test. (A) Maximum step reached. All animals that received stroke surgery had impaired reaching performance compared to shams. Those that received control therapy remained impaired for the duration of the study, while those receiving rehabilitation regained function on this test at 14 days. (B) Maximum number of pellets reached. All animals that received stroke surgery had impaired reaching performance compared to shams, which remained significant for the duration of the study. n=5/shams; 9/stroke groups; *= Stroke/Control significantly different from sham; #= Stroke/Rehab significantly different from sham; $p<0.05$.

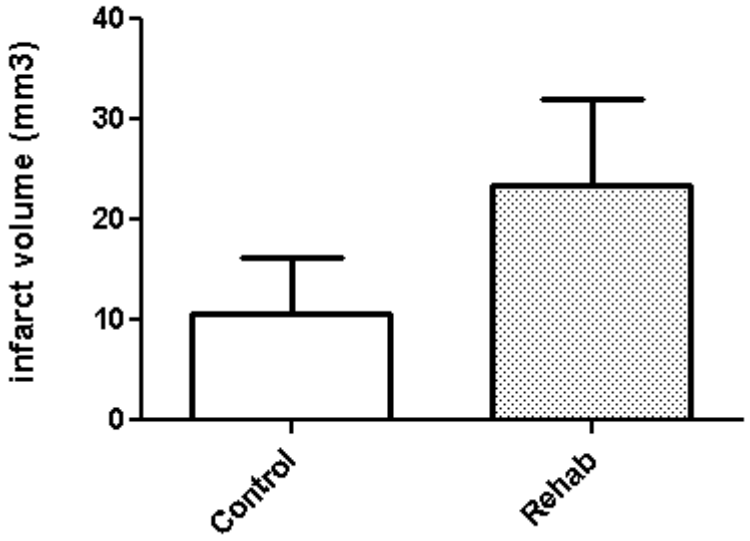


Figure 4.8. Infarct volume. Total infarct volume (mm^3) in stroke animals that received rehabilitation did not differ significantly from those receiving control therapy. n=9/group.

observing single labelled BDNF+ cells (subsequently referred to as "Other" cell type). Proportions were determined by calculating the percent of total BDNF that was co-localized with each cellular marker. Animals in the Stroke/Control group did not show altered BDNF expression compared to Shams. However, animals that received rehabilitation had significantly fewer BDNF+/NeuN+ cells ($p=0.021$), and significantly more single labelled "Other" BDNF cells ($p=0.043$; Figure 4.9-4.10). There were no significant differences were found in the contralesional hemisphere (data not shown).

4.3.4.2 Microglia

Microglia were visualized by labelling with anti-Iba1. Stroke caused a visible increase in the number of microglia present in the lesion border when compared to the Sham section (Figure 4.11), but there was no significant difference between the number of Iba1+ cells in the Stroke/Rehab group compared to the Stroke/Control group. No differences were found in the contralesional hemisphere.

4.3.4.3 Neuroblasts

Stroke/Control animals did not have significantly more Dcx+ cells in the ipsilesional SVZ, striatum, or cortex compared to the Sham group. Stroke/Rehab animals showed a significant increase in the number of Dcx+ cells in the SVZ and lesioned cortical areas ($F_{2,13}=3.95$; $p=0.050$) (Figure 4.12).

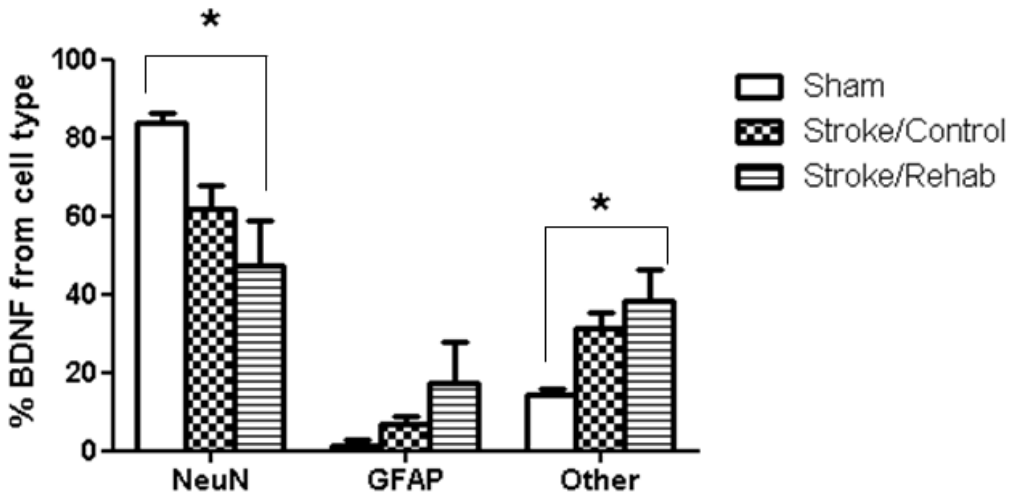


Figure 4.9. Ipsilesional BDNF expression. Triple labelling immunofluoresence was performed to determine the cellular origin of existing BDNF. Stroke/Control animals were not significantly different form either the Sham or the Stroke/Rehab groups. Animals that received rehabilitation had significantly less BDNF co-labelled with NeuN and significantly more non-co-labelled ('Other') BDNF (putatively microglia; see Discussion).

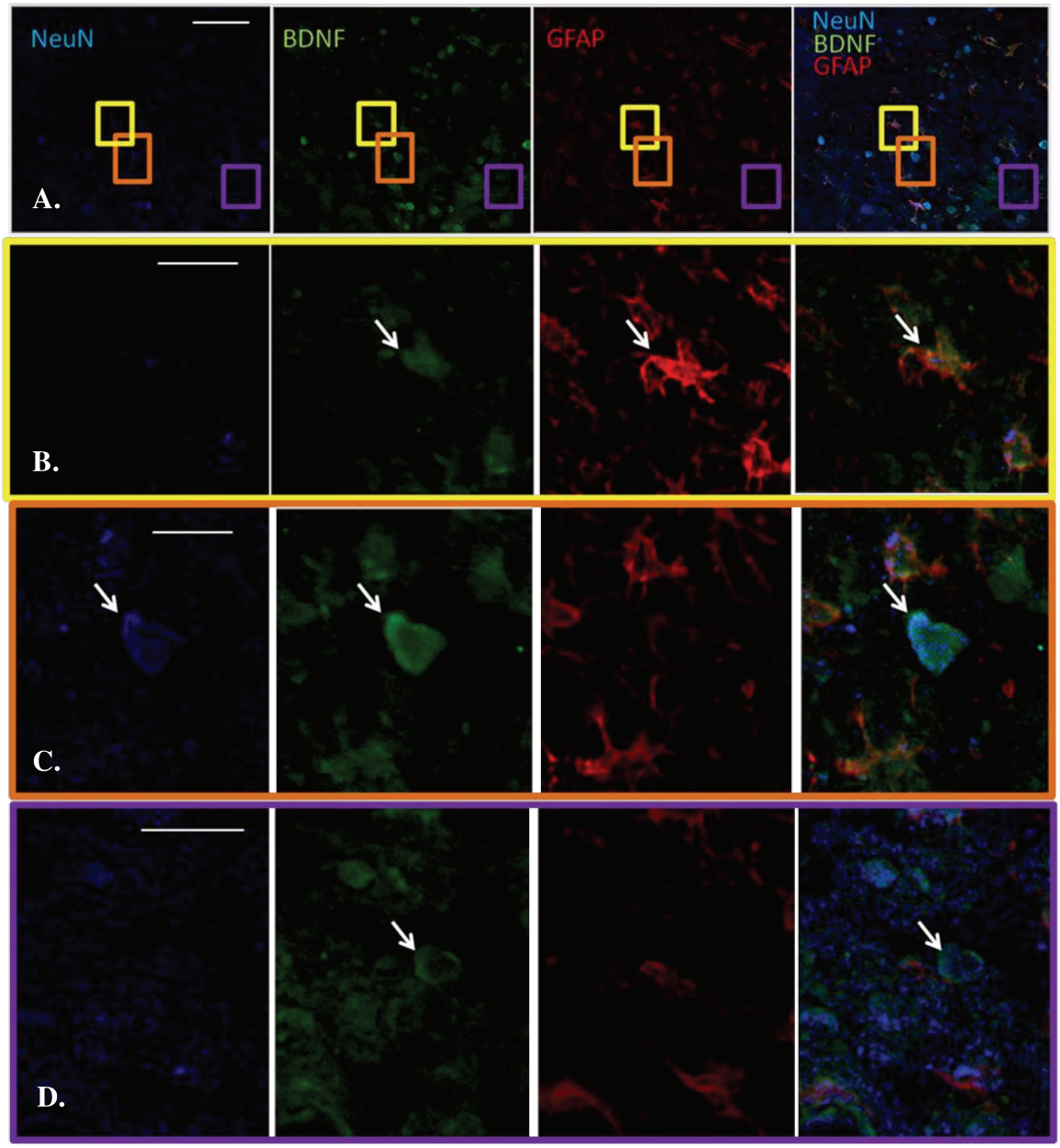


Figure 4.10. Cellular origin of ipsilesional BDNF. (A) Triple labelling immunofluoresence was performed to determine the co-localization of BDNF with NeuN and GFAP. Scale bar= 100 µm (B) Astrocytes expressing BDNF were observed as co-labelling of BDNF (green, arrow) with GFAP (red, arrow). (C) Neurons expressing BDNF were observed as co-labelling of BDNF with NeuN (blue, arrow). (D) Some BDNF was observed as non-co-labelled with either GFAP or NeuN, and subsequently referred to as 'Other' (arrow). (B-D Scale bar= 25µm)

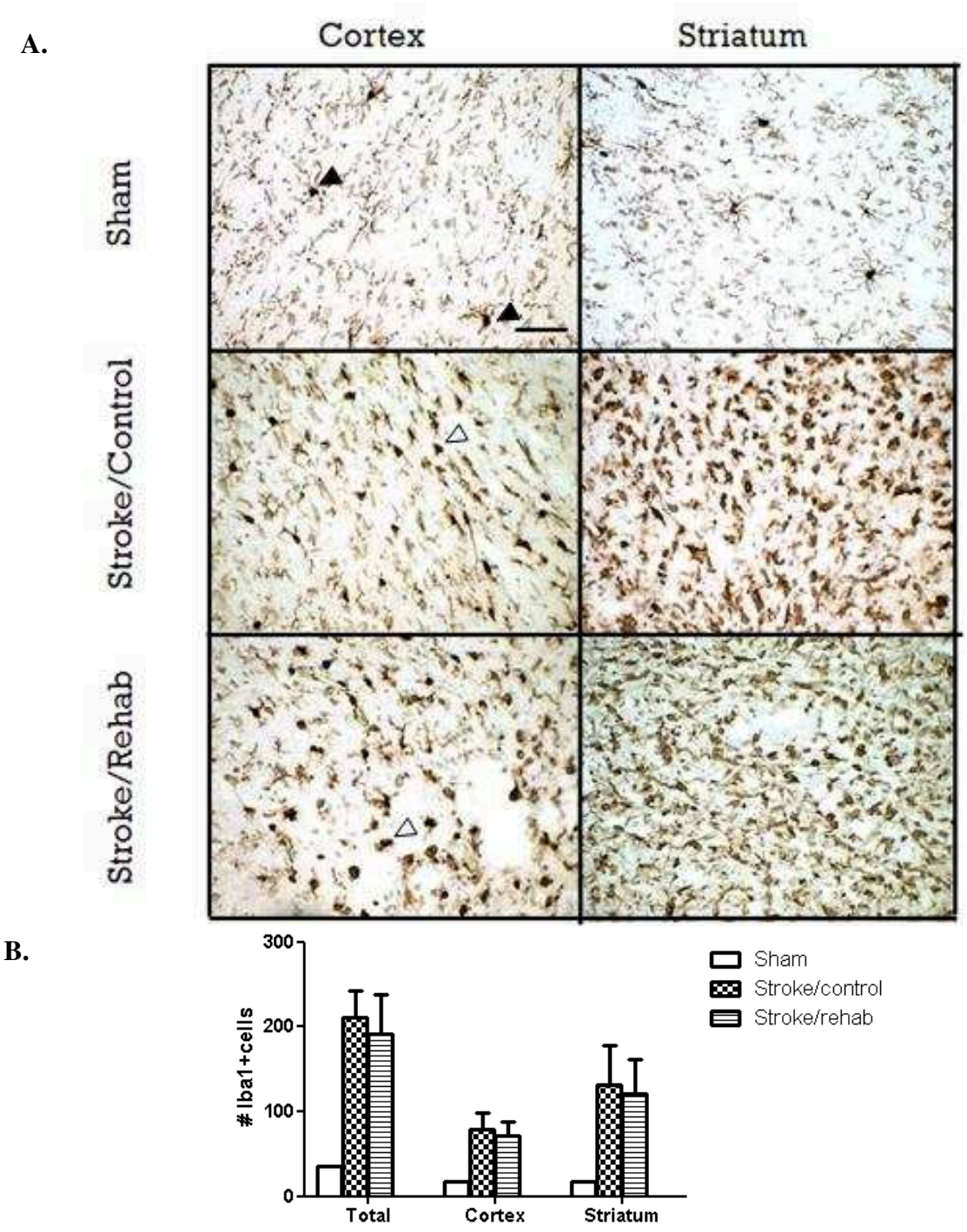


Figure 4.11. Presence of ipsilesional microglia. (A) Expression of microglia in Stroke groups was visibly increased compared to the Sham animal. In sham animals, the Iba1+ cells exhibited stellate morphology (solid arrowhead). In stroke animals, the Iba1+ cells were mostly ameboid, typical of activated microglia (empty arrowheads). Scale bar= 50 µm. (B) Rehabilitation did not affect the number of Iba1+ cells in any brain region. The white bar represents one Sham animal that was used for comparison, while n=4 for the two stroke groups, which were included in the analysis. *$p<0.05$.

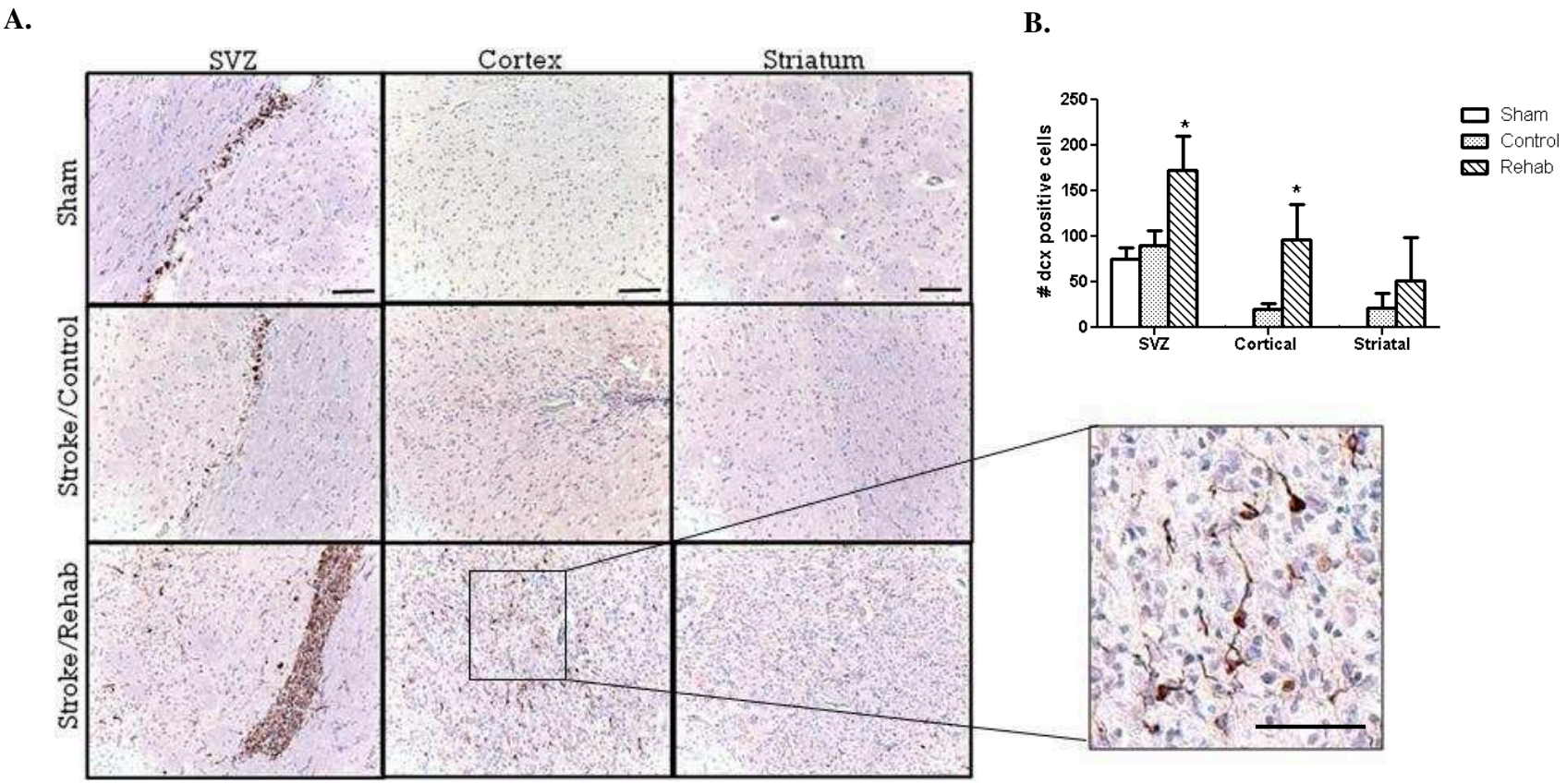


Figure 4.12. Ipsilesional Dcx expression (A) Normal expression of Dcx in sham animals (first row). Control therapy did not significantly increase the number of Dcx+ cells in the SVZ, nor in the ipsilesional striatum or cortex (second row and B). However, animals that underwent rehabilitation exhibited significantly more Dcx+ cells in the SVZ and in the ipsilesional cortex compared to either Sham or Control therapy animals (third row, and B), and the morphology of Dcx+ cells was long and thin (enlarged area). n=4/group; *p<0.05. Scale bar= 100 µm (enlarged area scale bar= 50µm).

4.4 DISCUSSION

Forced use of the paretic limb in stroke patients can enhance the recovery of motor function. However, animal models of forced use therapy have had varying success (Bland et al., 2000; Humm et al., 1998; Kozlowski et al., 1996). Encouragingly, post-ischemia exposure of rats to task-specific (Biernaskie and Corbett, 2001; DeBow et al., 2003; Maldonado et al., 2008) or non-specific (Johansson and Ohlsson, 1996; Ke et al., 2011; Marin et al., 2003; Ohlsson and Johansson, 1995) forelimb activity can improve functional outcome. Herein, a rehabilitation paradigm comprised of two voluntary components [generalized movement (activity ball) and task-specific movement (reaching box)] was evaluated (see Figure 4.1). This appetitively-motivated rehabilitation resulted in accelerated recovery of forelimb function on several tests (see Figures 4.5-4.7), without significantly affecting the amount of damage (see Figure 4.8). Examination of the effect of rehabilitation on BNDF expression revealed that while the proportion of total cells expressing BDNF was not different between groups at PSD 28, there was an increase in the proportion of non-neuronal, non-astrocytic cells expressing BDNF (see Figure 4.10). Further, there was a significant increase in the presence of Dcx+ cells in the ipsilateral SVZ and cortex of the Stroke/Rehab animals (see Figure 4.12).

Voluntary participation in activity ball movement therapy was similar throughout the course of this study, and averaged 160 m per day. This is in contrast to the declining intensity observed over the course of the study presented in Chapter 3 (see Figure 3.2). A potential explanation for this may be that the setup of the rehabilitation room was altered for the present study. The use of ANY-maze© tracking software required the

placement of one animal at a time into each rehabilitation arena, as opposed to the multiple animals approach used in Chapter 3. Perhaps this alteration resulted in increased incentive and curiosity of each individual animal for the duration of the study.

Prior to surgery, all animals used the intended contralesional limb to reach for pellets during the task-specific component of rehabilitation. In the days following surgery some animals behaved abnormally, for example by: 1) attempting to reach with the ipsilesional paw, 2) attempting to pull or knock over the reaching box to spill the pellets out, and 3) ignoring the reaching box. These behaviours mostly normalized (i.e. reaching with the contralesional paw resumed) within the first post surgical week. However, because these behaviours were occasionally observed, and because it often took multiple reach attempts to acquire pellets, the total number of pellets consumed is not indicative of the number of contralesional task-specific movements undertaken.

Interestingly, the Montoya staircase test demonstrated that animals receiving rehabilitation recovered reaching distance (see Figure 4.7A), but not the dexterity required to successfully consume the pellets in the apparatus (see Figure 4.7B). This could be due to the nature of the task-specific portion of the rehabilitation. In order to obtain pellets through the 1 cm reaching slot (see Figure 4.1B), animals were required to reach into the box (at a maximum depth of 3 cm), then could simply slide and scoop pellets out. Thus, the successful grasp and delivery of the pellet directly to the mouth was not required; rather, the pellets were obtainable once on the floor of the cage. Vaynman and Gomez-Pinilla (2005) suggest that different types of recovery may be specific to the form of rehabilitation implemented, supporting this explanation that the type of movement required for the present task-specific rehabilitation may underlie the recovery in maximum step reached but not in number of pellets eaten.

In the current study, testing took place on a weekly basis, rather than the daily and twice weekly testing in Chapters 2 and 3. This weekly testing schedule was chosen based on the gradual recovery profile observed from the studies presented in the previous chapters, which took place over three to four weeks. Daily testing was deemed needlessly frequent, and as highlighted in Section 1.8, repeated testing can itself be rehabilitative (Kleim et al., 2007). However, some resolution of recovery profile was lost because of the weekly schedule employed in the present study. For example, horizontal ladder running performance was only impaired in stroke groups on the first post surgical day, and by PSD 7 performance had recovered to sham levels in both groups. Thus, it was impossible to determine whether rehabilitation resulted in accelerated functional recovery in the first post surgical week, or rather, if both groups had recovered simultaneously. It is unclear why performance on the ladder test was not impaired for a longer period of time, as was seen in Chapter 3.

The volume of the infarct was not significantly affected by rehabilitation in this study, similar to previous reports (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Maldonado et al., 2008; Marin et al., 2003; Schabitz et al., 2004), although the mean volume in the rehabilitation group appeared greater than that of the control treatment group (25.7 ± 8.6 vs 10.6 ± 5.7 mm^3; $p=0.23$). Lesion exacerbation due to rehabilitation has previously been reported following forced use (Bland et al., 2000; DeBow et al., 2004; Humm et al., 1998) and enriched environment (Farrell et al., 2001) rehabilitation, perhaps related to timing and intensity of rehabilitation (Leasure and Schallert, 2004). Conversely, reduction in lesion volume has also been reported following exercise programs (Auriat and Colbourne, 2009; DeBow et al., 2003; Kim et al., 2005). The voluntary rehabilitation applied in this study likely exerts neural activation slightly

below the level required to result in significant damage, supporting the importance of low intensity, voluntary paradigms. It is possible that waiting until PSD 5 to initiate rehabilitation would have resulted in preservation of more tissue, as in Chapter 3.

BDNF expression was examined by immunofluoresence to ascertain whether rehabilitation resulted in a shift in the cellular origin of the BDNF being expressed. In the present study, rehabilitation caused a shift in BDNF production away from the neuronal phenotype and toward non-neuronal/non-astrocytic phenotype; a phenomenon that has been reported previously in the peri-infarct area (Béjot et al., 2011). In addition to neurons and astrocytes, other cell types that can express BDNF include microglia, endothelial cells, and ependymal cells. Because endothelial cells and ependymal cells are relatively easily identified based on location, morphology, and arrangement (Béjot et al., 2011), the observed morphology and location of the 'Other' BDNF cells (Figure 4.10D) supports the hypothesis that they are microglial (Béjot et al., 2011). Therefore, further investigation was made into the presence of microglia in the perilesional areas.

Historically thought to play a detrimental role in the injured brain, microglia are increasingly considered to have additional beneficial effects during post-ischemic neuroplasticity (Faustino et al., 2011; Iadecola and Anrather, 2011; Madinier et al., 2009). Madinier et al. (2009) reported that decreased acute microglial activation was associated with a long term down-regulation of markers of neuroplasticity, including BDNF. Narantuya et al. (2010) demonstrated that transplantation of human microglial cells following MCAo in the rat reduced ischemic deficits and cellular apoptosis. However, Hewlett and Corbett (2006) reported that administration of minocycline, an antibiotic with anti-inflammatory effects, improved functional recovery and reduced infarct volume. In the present study, visualization of Iba1+ cells showed that stroke

caused a large increase in the number of microglia in the perilesional areas, as reported by others (Auriat et al., 2010; Madinier et al., 2009). In sham animals, the Iba1+ cells all exhibited stellate morphology. In stroke animals, the Iba1+ cells were mostly ameboid, typical of activated microglia (Nowicka et al., 2008) (see Figure 4.11A). However, there was no difference in the number of microglia between the Stroke/Control and Stroke/Rehab groups. Therefore, rehabilitation may cause a shift in the role of local microglia (to express more BDNF), without affecting the actual microglial reaction. To further investigate this hypothesis, subsequent experiments should examine co-localization of BDNF and Iba1 (a process not possible in the present study due to insufficient tissue samples). In the absence of this confirmation, it is difficult to draw conclusions on the observed shift from neuronal to non-neuronal/non-astrocytic ("Other") sources of BDNF. The potential importance of this must not be overlooked, because the cellular source of BDNF has implications for potential target cell populations for post-stroke adjunctive therapies. If BDNF production is shifted significantly to microglial cells with rehabilitation, adjuvant therapies that inhibit microglial response could be counterproductive.

Lastly, this study showed an increase in the number of Dcx+ cells, indicative of migrating neuroblasts, in the SVZ and cortex of animals that received rehabilitation compared to control therapy. Thus, rehabilitation may increase the proliferation and/or survival of new neurons that migrate to damaged brain areas. It has been shown by several groups that brain injury and rehabilitation (Auriat et al., 2010; Briones et al., 2005; Leasure and Grider, 2010) result in reactive neurogenesis (Carmichael, 2008; Gu et al., 2000; Jiang et al., 2001; Jin et al., 2001; Kernie and Parent, 2010; Leasure and Grider, 2010; Parent et al., 2002; Wang et al., 2007; Xiong et al., 2010; Zhang et al.,

2001). However, a major limitation in this study was the use of Dcx as a marker of neurogenesis instead of the traditional BrdU injections. The reason for, and limitations of, this are discussed in Chapter 3 (see Section 3.4). Rehabilitation increased the expression of Dcx in the present study, and as in Chapter 3, the Dcx+ cells observed had characteristic morphology of migratory neuroblasts (long and thin, with few processes; Figure 4.12) and were located almost exclusively between the SVZ and the areas of damage. Further confirmatory studies specifically aimed at investigating the role of neurogenesis following rehabilitation, as well as long-term cell survival and integration, are warranted.

Modelling forced use using this appetitively-motivated paradigm appears to offer an alternative that overcomes some of the challenges associated with animal motivation and stress. This approach to voluntary forced use therapy results in modest acceleration of functional recovery using several tests, and may involve mechanisms related to BDNF expression and neurogenesis. Future studies should further investigate the involvement of microglia and neurogenesis in the post-ischemic neuroplastic process that result from this form of rehabilitation.

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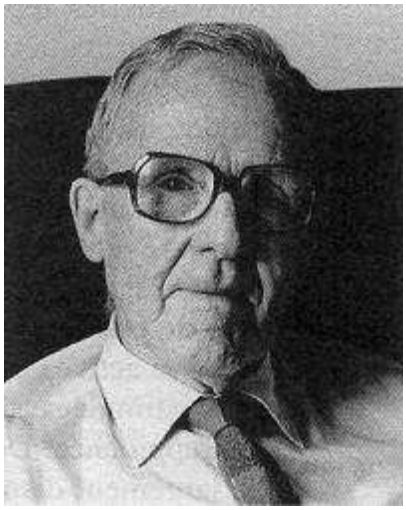
CHAPTER 5:

GENERAL DISCUSSION



"Mind can only be regarded, for
scientific purposes, as the activity of
the brain, and this should be mystery
enough for anyone"

-Donald Hebb (1904-1985)

picture from http://www.vetopsy.fr/comp/mem/mem_bn1.php

5.1 GENERAL OVERVIEW AND COMPARISON OF STUDIES

The discovery that the adult brain can undergo structural and functional changes following injury was truly a breakthrough in the field of brain repair. It has revolutionized the way in which we think about neurodegeneration and neurorehabilitation. While a few decades ago, rehabilitation revolved around learning compensatory function in order to optimize patients' quality of life, the focus has now shifted to driving positive, activity-dependent neuroplastic events to optimize recovery processes (Sandin, 2012).

Stroke is a complex disease with a plethora of causes, manifestations, and outcomes. Because the MCA is so commonly affected in stroke, and this artery supplies blood to cortical and striatal motor regions, impairment of limb function is a prevalent disability in survivors of stroke, and upper extremity impairment is common (Kelly-Hayes et al., 1998). The research described in this thesis aimed to address post-ischemic neuroplasticity following unilateral forelimb damage resulting from a temporary ischemic episode.

A promising rehabilitation technique for treating hemiplegic patients is CIMT. By forcing use of the impaired arm (by constraining the unaffected arm), recovery can be improved (see Section 1.7.1). The mechanisms by which this occurs are not completely understood, although neurotrophins have been implicated (Clarkson et al., 2011; Ke et al., 2011; MacLellan et al., 2011; Ploughman et al., 2007, 2009; Vaynman and Gomez-Pinilla, 2005). To better understand the effect of rehabilitation on neuroplasticity, animal models are required. The benefits of rehabilitation are greater when the patient is motivated to participate to the best of their abilities and at

increasingly progressive intensity (Carr and Shepherd, 2011). As such, clinical rehabilitation relies on patient willpower, clinician motivation, and family support. In animals, this presents a challenge. A novel approach intended to circumvent willpower and motivation issues while forcing use of the impaired forelimb was explored in the research described in this thesis.

An additional challenge when studying rehabilitation in animals is the choice of stroke model. Each of the commonly employed stroke models described in Chapter 1 (see Table 1.1) has a unique profile of benefits and disadvantages. The most commonly used model, MCAo, has the disadvantage of producing large and variable infarcts. While animals can survive surgical strokes that result in a large amount of damage, this may not represent a realistic model of the clinical condition, because humans are unlikely to survive such extensive tissue loss (Carmichael, 2005). Furthermore, the purpose of rehabilitation is to manipulate neuroplastic events in an activity-dependent manner. Neuroplasticity is known to occur at a cellular level, thus it is critical that sufficient brain tissue remains to form a substrate on which such changes can occur (Knieling et al., 2009; Teasell et al., 2005).

The specific objectives of this research were 1) to refine a rat model of focal ischemia to produce consistent, reliable infarcts to forelimb motor regions of the brain accompanied by quantifiable forelimb impairments, 2) to evaluate a novel voluntary model of forcing use of the impaired limb using behavioural and biochemical measures, and 3) to evaluate a refined version of the voluntary forced use model.

To address the first aim, an ET-1 intracerebral injection protocol was refined using a set of previously unpublished injection coordinates. This resulted in forelimb impairments lasting up to 3 weeks (see Section 2.3), and represented an improvement

over a previously established protocol in our laboratory. This refined protocol was then used for the remaining studies described in Chapters 3 and 4.

With respect to the second aim, initial evaluation of a novel forced use movement therapy revealed a slight acceleration in recovery profile in animals receiving rehabilitation. In this study, rehabilitation consisted of 30 minutes/day of low intensity movement in pet activity balls, initiated at PSD 5. While this rehabilitation did accelerate functional recovery to sham group level of performance (see Section 3.3), the results were not as pronounced as expected. Recovery was accelerated by as little as one day, for example in the forelimb postural reflex test (see Figure 3.6). Rehabilitation did not exacerbate damage, as indicated by the similarity in lesion volume between the control and rehabilitation groups (see Figure 3.9). However, despite the modest improvement in recovery, rehabilitation did not alter the proportion of cells expressing BDNF, $NOGO_A$, or the number of Dcx+ cells.

To further refine the rehabilitation protocol used in Chapter 3, an additional component was added to the therapy for the experiment described in Chapter 4. Inspired by the RTP administered as part of CIMT in the clinic, a repetitive skilled reaching exercise protocol was added to the rehabilitation. Additionally, because rehabilitation initiated on PSD 5 had not negatively affected lesion volume in Chapter 3, rehabilitation was initiated sooner in the following study (at PSD 3), in an attempt to maximize early neuroplastic opportunities while remaining in the 'safe' time frame accomplished previously. Further, testing of the modified rehabilitation study took place on a weekly, rather than daily or twice weekly, basis. Modified rehabilitation resulted in a more marked acceleration in functional recovery (between 1 and 3 weeks; see Section 4.3). This rehabilitation paradigm also caused a shift in the cellular origin of BDNF, and in

the number of Dcx+ cells, compared to sham control animals. Interestingly, the lesion volumes of animals receiving rehabilitation, while not significantly different from control animals, tended to be larger (Figure 4.8). Therefore, it appears that initiation of rehabilitation sooner than PSD 3 could lead to exacerbation of damage, which is consistent with previous findings (Bland et al., 2000).

Because of the differences in study designs presented in Chapters 3 and 4 (summarized in Table 5.1), it would be inappropriate to make direct comparisons between the respective rehabilitation regimens. Nonetheless, based on the use of some similar tests, and the lack of altered expression of neuroplastic markers observed in the initial rehabilitation study, it appears that the refined rehabilitation did result in the best recovery.

5.2 STROKE MODEL: CONSIDERATIONS

Chapter 2 described a variation of the ET-1 model that resulted in consistent damage to forelimb motor regions and reasonably long-lasting deficits (Hume, 2009; Leasure and Schallert, 2004; Schallert et al., 2000). Because of putative post-ischemic events that take place on a cellular level, it was important to use a stroke model that left sufficient intact neural tissue to act as a substrate for plasticity. Furthermore, the lesions were well-confined to the forelimb motor regions of the brain, reducing the possibility of confounding deficits.

While the variability between infarcts in each study was reasonable, there were large differences in the average infarct sizes between studies (Figures 2.6, 3.9, and 4.8). Efforts were made to preserve the integrity of reconstituted ET-1 within each study (see

	Chapter 3: initial evaluation of rehabilitation	Chapter 4: evaluation of refined rehabilitation
Duration of study	21 days	28 days
Rehabilitation exercise	30 minutes exercise ball	30 minutes exercise ball + 30 minutes repetitive task training
Initiation of rehab	Post surgery day 5	Post surgery day 3
Intensity of rehab	Declined over time	Remained constant (ball rolling); increased over first few days (pellet reaching)
Test battery	Forelimb placing tests (2) Horizontal ladder test Forelimb postural reflex Cylinder test	Forelimb placing tests (2) Horizontal ladder test Montoya staircase test
Test schedule	Daily (TFP, VFP, postural reflex); every 3-5 days (cylinder and ladder)	Weekly
Control groups	Surgical control (Sham); Rehabilitation control (Sham/Rehab)	Surgical control only (Sham)

Table 5.1. Contrasting rehabilitation studies. Summary of the major differences between rehabilitation studies performed in this thesis (for details, see Chapters 3 and 4).

Section 2.2.2). Thus, variations observed between studies may be due to ET-1 batch-to-batch variations, which have been reported by others (Soylu et al., 2012).

This research used exclusively male animals. This choice was based largely on previous work in the laboratory, as well as logistical challenges of using female animals in stroke research. Because estrogen and progesterone can affect infarct volume (Alkayed et al., 1998; Chen et al., 1999) and post-ischemic neuroplasticity (Woolley and McEwen, 1993), female animals must be monitored for estrous state throughout the experiment using cytological characterization of cervical smears (Maldonado et al., 2008), a labour intensive process. Further, it may be necessary to perform stroke surgeries at various times; unfortunately, the short storage life of prepared ET-1 would make this difficult. Thus, it is important to recognize that the studies presented herein should be replicated in female animals before making broad conclusions regarding efficacy. The general lack of confirmatory studies using both genders in preclinical research is believed to be one factor contributing to the demise of experimentally promising treatments when tested in the clinical setting (STAIR, 1999).

Another important point requiring emphasis is the age of the animals used in these studies. Because of a number of challenges surrounding the use of aged animals, this research focused exclusively on young adult rats (approximately 3-4 months of age at the time of surgery). Aside from additional housing costs, aged animals have a higher post-surgical mortality rate (Lindner et al., 2003; Pan et al., 2005), or require intensive post-operative care (Ryan et al., 2006). Furthermore, as rats grow larger, they become more difficult to handle properly and too large for some test apparatuses. These considerations make young adults a more attractive choice for short-term research projects. Nonetheless, the use of young animals is an increasingly criticized practice in

stroke research (Leasure and Grider, 2010; Lindner et al., 2003; Soleman et al., 2010; STAIR, 1999). As the majority of people who experience a stroke are elderly (Lloyd-Jones et al., 2009), using young animals may not provide an accurate portrayal of the physiological processes that take place in the average post-stroke brain. Li and Carmichael (2006) showed that the post-ischemic expression patterns of various markers of neuroplasticity were different in the aged brain compared to young adults (Carmichael et al., 2006). Thus, the data presented in this thesis should not to be extrapolated to an elderly population.

5.3 REHABILITATION: CONSIDERATIONS

Fundamental parameters of rehabilitation, including optimal duration and intensity, remain poorly understood. Some studies suggest that rehabilitation may be dose-dependent, with better outcome after more intense therapy (Huang et al., 2009b; Kwakkel et al., 1999). It also appears that a critical threshold of rehabilitation intensity must be reached to observe functional benefit (MacLellan et al., 2011).

It should be noted that like most post-stroke rehabilitation studies, the conditions in which animals were housed in the current work were considerably impoverished environments compared to the clinical setting, which further limits the translational relevance of results (Kleim et al., 2003). Even the most sedentary of stroke patients live in extremely complex environments, with regular cognitive, social, and environmental stimulation. This is in stark contrast to the institutional environment available to preclinical researchers, especially with the single-housing protocol used in the present research.

In Chapter 4, the use of ANY-maze© software allowed for the determination of the distance moved during the rehabilitation sessions. Analysis of a subset of 'early' 'middle' and 'late' time points revealed that animals consistently moved an average of 160 m/session. This is relatively low compared to other voluntary and forced use paradigms that have been explored. Generally, access to running wheels has been used to model voluntary exercise, and results in daily walking/running distances of 200-7000 m over the course of up to 23 hours (Ke et al., 2011; Maldonado et al., 2008; Mizutani et al., 2011). Forced exercise regimens, on the other hand, have involved daily distances of 82-1600 m, generally averaging 600 m over the course of an hour (Auriat et al., 2006; Kim et al., 2005; Leasure and Grider, 2010; Ploughman et al., 2007, 2009; Yanagita et al., 2007; Yang et al., 2003). Ke et al. (2011) measured corticosterone levels, indicative of stress, following voluntary running rehabilitation and found no difference from sedentary control animals, supporting the presumed 'mild' nature of voluntary forced use. Further, Yanagita et al. (2007) demonstrated an increase in the presence of corticotrophin-releasing hormone neurons following forced, but not voluntary, exercise. Mizutani et al. (2011) demonstrated that similarly mild voluntary rehabilitation (200-400 m/day) was sufficient to alter the expression of several proteins involved in neuroplasticity, supporting the hypothesis that the intensity observed in this thesis research could affect post-ischemic neuroplasticity. Without having directly measured stress levels, it is impossible to conclusively determine whether the paradigm of shorter periods of voluntary forced use used in this research evoked as little stress as other published voluntary paradigms. However, considering the fact that the rats were extensively and gradually familiarized with the activity balls prior to surgery, participation in the activity was optional, and animals could stop engaging during the

sessions if desired, it is likely that animals did not find the chosen activity level overly stressful.

This work did not include a 'dose'-dependent investigation of the rehabilitation treatment; rather, there was a set 30 minute interval during which animals were able to participate in each component of rehabilitation. The voluntary nature of the rehabilitation being evaluated made a dose-dependent approach challenging. Retrospective analysis of self-governed rehabilitation intensity revealed that there was little variability between individuals with respect to participation, but no attempt was made to determine, for example, whether allowing animals 60 minutes of activity ball time (rather than 30) would lead to further functional improvement. Further evaluation in this area would be interesting, considering the importance of at least a minimum intensity required to elicit benefit (MacLellan et al., 2011). The issue of dose/intensity also remains challenging for clinical researchers. Although CIMT intensity can be controlled to a degree (by prescribing a particular daily constraint duration) research into the importance of intensity has yielded mixed results (Page et al., 2005; Richards et al., 2006; Sterr et al., 2002).

Chapter 3 described an initial evaluation of only generalized voluntary forced use, using activity balls. In Chapter 4, a task-specific shaping exercise was added to the rehabilitation, intended to model the RTP component of CIMT (Sawaki et al., 2008; Wittenberg et al., 2003). However, in the interest of reducing the number of animals required for this experiment, there was no inclusion of a group that had each rehabilitation component (activity ball, reaching) separately. While the study in Chapter 3 allows for limited conjecture as to the value of activity ball alone, it is not possible to ascertain how much functional recovery might be observed by reaching alone. It may be

interesting to separate these components, to determine whether there is an additive or synergistic effect when combined. For example, Debow et al. (2003) found that following intracerebral hemorrhage, the application of either reaching alone or constraint alone (via the use of a constraint jacket) was not effective, while a combination therapy of both significantly improved recovery (DeBow et al., 2003).

5.4 BEHAVIOURAL TESTING: CONSIDERATIONS

As with all studies of post-stroke recovery, it is challenging to determine to what degree observed improvement represents recovery versus behavioural compensation. This is a concern that plagues both preclinical and clinical brain injury research (Duncan et al., 1992; Krakauer, 2005). When evaluating recovery, the perceived efficacy of a rehabilitation technique depends on the use of functional measures. However, behavioural outcome is the culmination of a number of processes that can be affected by numerous variables including recovery, compensatory strategies, stress, anxiety, and general well-being. This creates a complicated web of interactions that may confound results.

To reduce the confounding factor of compensation, due consideration must be given to the use and interpretation of appropriate tests. For example, in the clinic, a commonly used assessment of deficit and recovery is a scale of performance on activities of daily living (ADL) (Jorgensen et al., 1995; MacKay-Lyons and Makrides, 2002; Ramasubbu et al., 1998). However, a patient who has learned to perform activities of daily living by using the unaffected arm can exhibit an improved score on this test despite the absence of functional recovery in the impaired arm. To address this, the

present research consisted of a battery of tests in each study, targeting a variety of impaired behaviours (i.e. sensorimotor reflex, postural positioning, coordination, spontaneous movement, skilled reaching). These tests encompassed both similar and different movements to those induced by rehabilitation [i.e. ladder walking (similar to ball rolling) and staircase reaching (similar to repetitive pellet reaching)]. In the study presented in Chapter 4, performance on the Montoya staircase test (with respect to 'number of pellets consumed') did not return to control level (see Figure 4.7B). However, animals showed improved performance on other assessments used (tactile stimulated placing test, vibrissae stimulated placing test, and maximum step reached on the Montoya staircase). Thus, it is plausible that lack of recovery on 'number of pellets consumed' was due to a more profound deficit in the complex function required for that task, rather than the animals exhibiting compensation on all other tests.

Nonetheless, it is challenging to determine the best test battery to use for any study that will be appropriately sensitive to the nature of the deficit and also reduce the opportunity for compensatory performance. Several other forelimb functional tests popular in stroke and rehabilitation research were not used in this research, including adhesive removal (Leasure and Grider, 2010; Müller et al., 2008; Schallert et al., 2000; Soleman et al., 2010), beam traversing (Windle et al., 2006; Zhao et al., 2009), and single pellet reaching (MacLellan et al., 2006). Some tests that were used (e.g. the cylinder test in Chapter 3, and the ladder test in Chapter 4) were not sufficiently sensitive for this purpose. However, for each study, the test batteries were carefully chosen with the expectation that they would be appropriate based on previous experience and existing literature. Unfortunately, the use of too many or overly technical tests, frequent testing periods, and tests too similar to the rehabilitation being evaluated

can result in inadvertent rehabilitation (i.e. the performance of the test battery confounds the post-ischemic recovery process being investigated) (MacLellan et al., 2006), necessitating the use of concise test batteries. For future studies of this nature, the continued use of the forelimb placing tests (based on ease of use and sensitivity to significant changes) and the staircase task (based on the more profound and long-lasting deficit on this test, perhaps making it sensitive and suitable for even longer-term studies of rehabilitation) should be implemented. Also, more work should be done to investigate the discrepant ladder test results, which appeared sufficiently sensitive to injury in Chapter 3 (see Figure 3.8) but not Chapter 4 (see Figure 4.6). Other tests, such as those highlighted above and used by other investigators, have yet to be evaluated using the present surgical or rehabilitation protocols.

An alternative approach to the measurement of functional outcome would be to implement more qualitative assessments. Rather than analysing whether the animals can do something (e.g. reach for pellets on the staircase), this approach would involve detailed analysis of how the animal performs a task. For example, qualitative analysis of limb movements during paw reaching (Gilmour et al., 2004; MacLellan et al., 2006; Whishaw, 2000) may provide more information about the nature of how animals receiving rehabilitation use the impaired forelimb compared to controls. Because such qualitative analyses have not yet been standardized for the staircase test (MacLellan et al., 2006), a separate, labour-intensive single pellet reaching task would be required (Auriat and Colbourne, 2009; MacLellan et al., 2006; Maldonado et al., 2008; Whishaw, 2000).

5.5 MARKERS OF NEUROPLASTICITY: CONSIDERATIONS

5.5.1 Growth-associated proteins

The use of growth factors as therapeutants following stroke is generally hindered by poor permeability of the blood brain barrier, thus limiting bioavailability. Research into the use of delivery vectors (Wu, 2005) aims to overcome this obstacle; however, treatments that result in increased endogenous expression remain a promising alternative.

The study presented in Chapter 3 demonstrated that surgical stroke resulted in an increase in the proportion of cells expressing BDNF in perilesional tissue (see Figure 3.10), similar to other reports (Béjot et al., 2011a, 2011b; Johansson, 2000; Madinier et al., 2009). However, there was no increase in contralateral BDNF expression detected, contrary to Kim et al. (2005). In that experiment, BDNF expression was examined at PSD 16, rather than PSD 21. Due to the dynamic temporal profile of post-ischemic neuroplastic gene expression (see Section 1.4.3), it is possible that the rehabilitation evaluated herein caused earlier contralesional changes that were not detected on post mortem analysis. The potential impact of timing is further supported by the results described in Chapter 4. In that experiment, examination at PSD 28 revealed that there was no longer a change in the ipsilesional proportion of cells expressing BDNF. Thus, stroke may increase ipsilesional BDNF expression until sometime between PSD 21 and 28.

The lack of an increase in the proportion of BDNF-expressing cells in Chapter 4 led to interest into whether or not there was a change in the cellular origin of the BDNF

being expressed in animals in the rehabilitation group. To determine this, co-labelling was performed to examine whether the BDNF-expressing cells were neurons, astrocytes, or neither. Interestingly, animals receiving rehabilitation had an increased expression of BDNF in cells that were not immunopositive for NeuN (neurons) or GFAP (astrocytes). As discussed in Section 4.4, the most likely source of 'other' BDNF is microglia. Unfortunately, confirmatory analysis was not possible, due to a lack of remaining tissue samples.

The BDNF antibody used in the current work, Millipore catalogue # AB1779 rabbit anti-BDNF, does not allow distinction between pro-BDNF and the mature form of the neurotrophin. Therefore, there may be changes in the amount of either form that were not detected in the present research. Ultimately, discrimination of the two forms may be important, since the beneficial effect of this neurotrophin is believed to be restricted to its mature form (Greenberg et al., 2009; Lu et al., 2005). Antibodies specific to pro-BDNF are commercially available (e.g. Millipore catalogue #AB9042) and would be required for such an investigation. Recent work by Madinier et al. (2013) showed that peri-lesional expression of the two forms of BDNF are differentially induced following photothrombosis, wherein the level of mature BDNF shows an early increase, followed later by an increase in levels of pro-BDNF (Madinier et al., 2013).

While BDNF is a commonly studied neurotrophin in rehabilitation research, it is certainly not the only interesting candidate for involvement in rehabilitation-induced neuroplasticity. A growing list of growth factors have been implicated in post-ischemic neuroplasticity, including FGF-2, epidermal growth factor (EGF), IGF-1, erythropoietin (EPO), stem cell factor (SCF), vascular endothelial growth factor (VEGF), GDNF, and NGF (Greenberg and Jin, 2006; Horinouchi et al., 2007). The following section will

highlight some interesting findings with respect to post-stroke growth factors; for an in-depth review see Greenberg and Jin (2006).

As stated in Section 1.4.3, NGF belongs to the same family of neurotrophins as BDNF (Lu, 2003). Interestingly, like BDNF, NGF is also released as a result of cellular depolarization (Zafra et al., 1990), making it a logical candidate for involvement in post-ischemic neuroplasticity. Indeed, two groups recently reported that ischemia followed by forced or voluntary exercise resulted in upregulation of NGF (Matsuda et al., 2011; Mizutani et al., 2011). Unfortunately, NGF is clinically associated with hyperalgesia (Greenberg and Jin, 2006), which may hinder any therapeutic potential.

Established functions of GDNF include neurite outgrowth and interference with apoptotic and necrotic pathways (Abe, 2000; Kitagawa et al., 1998; Zhang et al., 2001b), potentially important in the post-ischemic brain environment. So named because of its original isolation from cultured glial cells, expression of GDNF is also induced in neurons following MCAo and photothrombotic stroke (Abe and Hayashi, 1997; Horinouchi et al., 2007). Post-stroke administration of GDNF is neuroprotective (Kitagawa et al., 1998; Zhang et al., 2001b, 2002). However, to date, the effects of rehabilitation on GDNF expression have not been examined.

5.5.2 Growth inhibiting proteins

The effect of the current model of rehabilitation on growth inhibition was also investigated by examining the expression of $NOGO_A$. Because $NOGO_A$ has been implicated in post-ischemic recovery (Fang et al., 2010; Papadopoulos et al., 2002), it is plausible that rehabilitation that results in functional improvement may act in part by

reducing the expression of this growth-inhibiting protein. However, in the study described in Chapter 3, there was no difference in the proportion of $NOGO_A$+ cells in either stroke group. Previous work has shown that the expression of $NOGO_A$ (measured by Western blot analysis) is higher in animals 7-14 days following stroke, although an extended time period was not analysed (Li and Carmichael, 2006). As discussed in the previous section, it is possible that by the time point chosen in Chapter 3 (PSD 21), $NOGO_A$ expression had normalized. Because expression was not different at that time, $NOGO_A$ analysis was not performed in the study presented in Chapter 4.

The temporal expression profiles of several growth inhibitors have been characterized following MCAo (Carmichael et al., 2005; Li and Carmichael, 2006). There was an initial upregulation of several genes involved in growth inhibition in the first 14 days following injury, which were generally no longer significantly increased by PSD 28 (e.g. NOGO, ephrins A5 and B1) (Carmichael et al., 2005). Thus, there may be a critical time period after the first 2 weeks post-stroke in which expression of $NOGO_A$ changes significantly, but normalizes by PSD 21. However, it must be noted that the measurement of mRNA does not necessarily correlate with the expression of functional proteins, and 'mismatches' between mRNA levels and protein expression have been reported previously (Li and Carmichael, 2006).

5.5.3 Doublecortin

While it has long been recognized that many adult tissues have the capacity to be renewed through the production of stem cells, a major breakthrough in the study of neurodegenerative disease was the discovery that the adult brain—previously thought to

cease cell renewal following development—continually produces new cells (Altman, 1962; Eriksson et al., 1998). Neural SCs are constantly being formed in SGZ and the SVZ, adjacent to the lateral ventricles. Under normal conditions, SCs from the SVZ migrate to the rostral olfactory lobe along the rostral migratory stream (RMS) (Lois and Alvarez-Buylla, 1994; Momma et al., 2000). Interestingly, following brain damage such as ischemia, increased neurogenesis can be observed (Arvidsson et al., 2002; Jin et al., 2001; Liu et al., 2009; Zhang et al., 2001a). SCs originating from the SVZ are re-routed from the RMS toward the ischemic regions (Hicks et al., 2009; Ohab et al., 2006; Parent et al., 2002), potentially representing an endogenous repair mechanism. Analysis of Dcx+ cells in Chapters 3 and 4 suggested that the ET-1 stroke model promotes neurogenesis (see Figures 3.12 and 4.12; for discussion of the limitations of this analysis, see Section 3.4) and that the modified rehabilitation regimen used in Chapter 4 further increased the number of migrating neuroblasts (see Figure 4.12).

Chapter 3 demonstrated a mild functional benefit of rehabilitation without affecting the number of Dcx+ cells (see Figure 3.12). Chapter 4 revealed a more robust acceleration in functional recovery and an increase in the number of Dcx+ cells. This suggests that the migration of new neurons to damaged tissue may have a potentially positive impact on functional recovery. Nonetheless, neurogenesis is ultimately the culmination of several equally important cellular events: proliferation, migration, differentiation, and ultimately circuit integration and survival (Zhang and Chopp, 2009), processes not fully addressed in this work. Furthermore, there is some evidence that ischemia can lead to aberrant neurogenesis, raising concerns about the contribution of newly born neural cells to functional impairments (Niv et al., 2012). This aspect of rehabilitation would be interesting to pursue in greater detail.

5.6 TRANSLATIONAL CONSIDERATIONS

While animal models remain critical to basic stroke research, there are limitations in the translation of research findings to clinical practice. As with other experimental conditions, stroke models represent a simplified version of an extremely complex human condition. Gender, age, pre-stroke condition, stroke severity, and co-morbidities are highly variable in the clinical population, but tightly controlled in experimental studies. The amount of damage and subsequent severity of impairment are also disjointed between the animal and human population, as animals both require and can survive more severe neurological damage in order to produce detectable functional deficits.

With the plethora of variables to consider at each step, modelling rehabilitation presents a major challenge and remains fundamentally different from rehabilitation in the clinical setting. Stroke patients undergoing CIMT receive supervised, assisted, and highly motivated treatment from trained experts. Experimental rehabilitation is largely hands-off, and animals are more difficult to motivate. Decisions must be made to best represent the validity of a model with respect to a particular aspect of the therapy. Such decisions may result in compromises in aspects of the therapy, for example intensity, in order to address stress and behavioural pressure.

A further difficulty in attempting to translate findings from animal rehabilitation studies is that the number of exercise repetitions is typically quite high in animals compared to human stroke survivors. As shown in Figure 4.4B, for example, animals were reaching 70-80 pellets per 30 minute session, similar to the degree of engagement reported by others (DeBow et al., 2003; Maldonado et al., 2008). However, an

observation of clinical rehabilitation revealed that patients are required to perform considerably less (on average, 32 repetitions of an exercise during a session, divided between 2-4 separate tasks) (Lang et al., 2010). Thus, it is possible that current practices in rehabilitation are administering very low levels of rehabilitation (Nudo, 2011), an especially important consideration as there appears to be a threshold of rehabilitation below which functional improvements are not observed (MacLellan et al., 2011). A proof-of-concept study recently concluded that it is feasible to attain repetitions in human stroke patients that resemble those reported in the animal literature (Birkenmeier et al., 2010).

Because the role of rehabilitation is to facilitate recovery processes after infarct development (rather than to provide neuroprotection), it is tempting to assume that it may translate to other neural injuries that result in forelimb hemiplegia. It is important to note that the mechanisms of injury following aspiration lesions and stab wounds differ substantially from those post-ischemia, with respect to the dynamics and distribution of inflammation, apoptotic cell death, free radical damage, and post-injury axonal and dendritic growth (Carmichael, 2006; Gonzalez and Kolb, 2003; Kempermann et al., 2000). Nonetheless, clinical examinations of CIMT in other neuropathological paradigms have been promising. Following traumatic brain injury, hemiplegic patients responded well to CIMT (Shaw et al., 2005) and positive results have been reported following the use of CIMT in cerebral palsy patients (Huang et al., 2009a). Until the mechanisms underlying treatment induced recovery are fully understood, it is difficult to determine what other injury models might benefit. Perhaps variables like lesion size, remaining tissue, and patient demographics are more influential than the nature of the lesion.

5.7 FUTURE DIRECTIONS

Future investigations should first attempt to further optimize the rehabilitation paradigm. Both rehabilitation studies presented in this thesis resulted in a statistically significant acceleration in functional recovery to control levels in a number of functional tests, supporting the use of voluntary forced forelimb use in a rat model. However, the magnitude of the effect was lower than expected, often only accelerating recovery by as little as one or two days (Chapter 3) or a week (Chapter 4). As highlighted in Section 5.3, the duration of rehabilitation sessions was relatively constant throughout the studies. Some others have used rehabilitation paradigms that increase in intensity over time (Auriat et al., 2006; Maldonado et al., 2008; Ploughman et al., 2009), with varying success. In clinical rehabilitation, higher intensity therapeutic practice has a greater impact on task performance (Kwakkel, 2006), though at a certain point, increased intensity induces fatigue and counteracts the positive effect (Sterr and Saunders, 2006). Thus, the determination of how increasing the intensity of the rehabilitation, either over the duration of the study or as a constant delivery of higher intensity, would be of interest in exploring this model further. As previously described, a task-specific component was added to the voluntary forced use paradigm in Chapter 4, to more closely model clinical CIMT (see Section 4.2.3.2). The research presented in this thesis did not investigate separate general and task-specific components. Doing so may prove useful for investigating varying intensities of each component separately.

As highlighted in Section 5.2, the use of young adult rats in stroke research is becoming increasingly criticized (Leasure and Grider, 2010; Lindner et al., 2003; Soleman et al., 2010; STAIR, 1999), and the aged brain responds differently than the

young adult brain following stroke (Li and Carmichael, 2006). Thus, it would be interesting to determine if the present approach to rehabilitation can accelerate functional recovery in older rats. While several logistical challenges would need to be overcome (described in Section 5.2), these barriers have hindered the implementation of this important variable for too long, likely contributing to the lack of translational value frequently observed in preclinical stroke research (STAIR, 1999).

Evaluation of the efficacy of this model of therapy should examine the persistence (or further development) of functional recovery beyond the end of treatment. Clarke et al. (2009) showed that environmental enrichment rehabilitation resulted in a lasting functional benefit beyond the completion of treatment. Similarly, Taub et al. (2006) reported that following CIMT treatment, patients retained functional improvement at a four week follow-up assessment. However, some clinical rehabilitation regimens may show improvements in motor function that are lost upon discontinuation of the therapy (Kleim et al., 2007). The long-term effects of the rehabilitation examined in this thesis should be determined through future studies.

Because of the heterogeneity of ischemic injury and subsequent repair mechanisms, a promising strategy for developing stroke treatments is to combine therapies (Ratan and Noble, 2009). For example, adjuvant treatment with a pharmacotherapy or natural health product along with voluntary forced use rehabilitation could prove more effective than either treatment alone. Enriched environment (Ramic et al., 2006), forced exercise (Sakakima et al., 2012), and skilled reaching (Fang et al., 2010; Windle and Corbett, 2005) rehabilitation have been shown to increase the efficacy of D-amphetamine (Ramic et al., 2006), S-nitrosoglutathione (Sakakima et al., 2012), fluoxetine (Windle and Corbett, 2005), and NOGO receptor antagonist (Fang et al.,

2010) treatments, resulting in better recovery than either the rehabilitation or drug alone. This suggests that activity-dependent rehabilitation may engage certain instructive cues that optimize the neuroplastic changes occurring in the post-stroke brain microenvironment. Thus, following further optimization of the voluntary forced use movement therapy, it may prove to be a useful adjuvant to treatment with promising pharmaceuticals and nutraceuticals.

5.8 CONCLUSIONS

Animal models will never perfectly mimic the human condition, but are intended to guide scientific understanding and provide insight into specific processes involved in post-ischemic recovery. As such, several studies have already been critical in identifying processes that may underlie the functional improvements resulting from CIMT. The novel voluntary forced use movement therapy rehabilitation model developed and evaluated in this thesis adds to this body of literature. This model resulted in accelerated functional recovery following ET-1 induced lesions to forelimb motor brain regions, while circumventing animal stress and behavioural pressure issues associated with some other forced use models. Further optimization may lead to increasingly robust acceleration in recovery.

The importance of post-stroke activity-dependent neuroplasticity must be recognized. Stroke patients usually have diminished capacity for exercise (MacKay-Lyons and Makrides, 2002), thus an understanding of post-stroke activity-dependent mechanisms, and how they might be enhanced, could prove crucial in developing improved therapies. The remaining challenge is to ensure that experimental research is

effectively translated to clinical practice in order to capitalize on existing knowledge, identify common findings, and ultimately ensure positive collaborations toward improving post-stroke recovery.

"[The brain] is, without doubt, the last frontier of science"

-Eric Kandel

5.9 REFERENCES

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